THE ROLE OF FIXED AND MIGRATORY CELLS

IN IMMUNOLOGICAL REACTIONS

A THESIS

submitted for the degree of

DOCTOR OF PHILOSOPHY

in the

AUSTRALIAN NATIONAL UNIVERSITY

by

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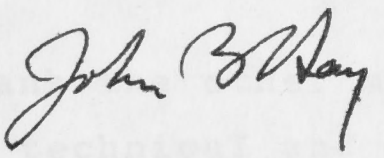
December, 1970.

Statement

The experiments reported in this thesis were done by myself. I acknowledge the help of Dr. E. Adams with the assay of lysosomal enzymes, Dr. A. Yamashita with some of the cell separations and Dr. B. Morris with the identification and ultrastructural analysis of antibody-forming cells.



Acknowledgements

The work carried out in this thesis was done in the Department of Experimental Pathology, John Curtin School of Medical Research. I am grateful to the Australian National University for the scholarship and to Professor F.C. Courtice for the use of the facilities of his department.

My appreciation goes to Professor Bede Morris, not only for his supervision and assistance but also for sharing with me some of his insight into the ethics and standards of science and scientists.

I would also like to thank the other members of the departmental staff - academic, technical and clerical, for their advice and assistance.

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PREFACE

Immune reactions as they occur in mammals cannot be described either simply or complexly in terms of immunoglobulin synthesis or clonal selection. Any immune response is the end result of a whole sequence of interacting physiological and pathological events which involve not only the cells of the reticuloendothelium but the lymphatic and blood vascular systems and frequently non-lymphoid organs and tissues as well.

Because the physiology of the immune response has not occupied the attention of immunologists, the highly significant features of inflammation, cell migration, changes in vascular permeability and tissue destruction that occur in many immune reactions have tended to be set apart from the phenomenon of antibody synthesis and the development of the hypersensitivity state. This restricted viewpoint has prevented a proper appreciation of the character of the immune response as it normally occurs. Immune reactions are the result of interacting systems and not just interacting cells. In these terms an understanding of the magnitude of the immune response and its dynamic character can only be appreciated in experimental situations where physiological and pathological consequences can be seen in their true perspective.

The experiments described in this thesis aim to show some of the features of various types of immune responses in their physiological context. An attempt has been made to integrate the changes that occur in non-lymphoid tissues of the body with the changes that occur in the afferent and efferent lymph and in the regional lymph node itself. The approach has been essentially from the standpoint of lymphatic physiology, the experiments being designed to establish quantitative estimates of immune reactions in terms of cell migration, cell proliferation and differentiation, as well as in terms of the changes that occur in the functioning of the lymphatic system itself. These physiological events have been correlated with the synthesis of specific antibody protein by individual cells of the migratory lymphoid population and with certain other aspects of their metabolism.

CHAPTER I

INTRODUCTION

Introduction

The effector agents in immunological reactions are cells of the reticulo-endothelial system. These cells are distributed widely throughout the body and can be found in all tissues. There is however, a particular association between both fixed and mobile elements of the reticulo-endothelium and the lymphatic vessels that has a particular relevance for the immune response.

In the course of the development of a cardiovascular system which enabled oxygen and nutrients to be delivered to the tissues under considerable pressure, a range of osmotically active protein molecules also appeared. These molecules assist in retaining water within the vascular compartment and thus maintain the circulating blood volume constant. The characteristics of the blood capillary membrane, however, are such that small numbers of protein molecules are continually escaping from the blood stream and entering the tissue spaces. These molecules are precluded from passing directly back into the circulation; in order to continue to subserve their osmotic function they must be removed from the tissues and transported back to the blood by a specialized system of absorbing vessels, the lymphatics. The principal feature of the lymphatic vessels is their unique capacity to take up proteins and other macromolecular or particulate material from the tissue spaces and they perform this function in practically all organs and tissues of the body.

Not only are the lymphatic vessels concerned with the removal of plasma proteins from the tissues as a normal physiological activity, but also because of their nature, they are invariably implicated in taking up any foreign, antigenic matter (macro-molecules, bacteria or viruses) that may enter the body through breaches in the skin or mucous membrane.

There are present along the lymphatic vessels, specialized aggregations of reticulo-endothelial cells organized into lymph nodes. These provide sites where

phagocytic cells remove and concentrate any foreign matter present in the lymph stream. In addition, in these organs there are vast numbers of lymphocytes, reticulum cells, plasma cells and other cell types with immunological capabilities which have the opportunity to come into contact with antigens there and initiate an immune response. In such an arrangement in which the specialized absorbing functions of the lymphatic vessels are linked with the removal and sequestration of foreign material in situations where immune effector cells congregate, a highly efficient system has evolved for dealing with naturally occurring antigenic challenges.

The capacity of the lymphatic system to deal with foreign material is greatly facilitated by the extraordinary mobility of the lymphocyte. This central cell of the immune response is capable of migrating from the blood stream into almost any situation in the body. Whilst these cells migrate most obviously through the substance of the lymph nodes, they can also pass into sites where antigens are held outside the fixed lymphoid tissues and initiate an immune reaction locally. Once outside the blood stream, these cells depend on the lymphatic system to provide the means to enable them to move from the tissues into the lymph nodes and back to the blood. There exists then, a population of migratory immune effector cells, associated with a specialized system of absorbing vessels which takes up and transports antigenic material. The lymph stream thus affords an opportunity of monitoring aspects of the immune response which involve antigen, effector cells and the end products of their interaction.

The Distribution of Lymphocytes

Lymphocytes are found not only in efferent lymph coming from lymph nodes where they are the characteristic cell, but also in afferent lymph from a wide variety of tissues and organs where they are found in smaller numbers usually associated with macrophages. Lymphocytes are also

prevalent in connective tissues of all organs and in the circulating blood (Yoffey and Courtice, 1956). Although the ubiquitous nature of the lymphocyte was an accepted fact as early as the beginning of the twentieth century, the origin, fate and function of these cells was not appreciated until the last 15 years or so, and up until this time the lymphocyte was an enigma of pathology. Recently, quantitative, physiological studies have shown that large numbers of lymphocytes are continually leaving the blood vessels and entering the extra-vascular compartment of tissues. Once outside the blood vessels, it appears that the only way lymphocytes can escape from the tissues (except in the bone marrow and the spleen) is by way of the lymph stream.

It has been calculated that the daily output of lymphocytes in the major lymph ducts of the body is enough to replace the total number of lymphocytes in the circulating blood of most animals 5 - 10 times (Reinhardt, 1964). The extent of the lymphocyte traffic through the different tissues has been measured in conscious sheep by collecting quantitatively the lymph draining from a variety of organs and tissues. In addition comparisons have been made between the numbers of cells found in lymph before and after it passes through lymph nodes, thus providing a comprehensive account of the traffic of lymphocytes in the sheep. (Smith, McIntosh and Morris, 1970).

In fixed lymphoid tissues where lymphocytes are concentrated together with phagocytic cells of the reticulo-endothelial system there appears to be a type of specialized vasculature which permits the passage of lymphocytes from the blood on a more extensive scale than normally occurs in other tissues. These so called post-capillary venules, which have a characteristic type of high endothelium appear to accomodate this extensive traffic in normal lymph nodes (Gowans and Knight, 1964; Marchesi and Gowans, 1964) and in the Peyer's patches of the gut (Schoefl, 1970). Hall and Morris (1965a) showed that normally, 95 per cent of the lymphocytes leaving the popliteal lymph node in

efferent lymph came from the blood and these cells pass presumably for the most part through the post-capillary venules. The number of cells migrating through a single popliteal lymph node amounts to between 5×10^7 to 10^8 cells per hour. In addition to these special arrangements for accomodating an extensive traffic of lymphocytes from the blood, lymph nodes are also characterized by other structural features which facilitate the handling of antigenic material.

Changes in Lymph Nodes Following Antigenic Challenge

Lymph nodes are primarily aggregations of lymphocytes, transformed cells, plasma cells and phagocytic cells situated at intervals along lymphatic vessels. To consider the relationship between the various types of cells and the significance of the structural components of a lymph node it is convenient to trace the sequence of events occurring in an immune response within a lymph node.

A naturally occurring antigenic challenge almost invariably reaches a mammalian lymph node via the afferent lymphatics. These vessels penetrate the lymph node capsule around the periphery of the cortex. In the cortex there occur lymphoid follicles or dense accumulations of lymphocytes which extend up to the cortico-medullary region of the node where the post-capillary venules are situated. It seems likely that when an antigen localizes in a lymph node, increased numbers of lymphocytes leave the blood and are recruited into the node (Hall and Morris, 1963). This event leads to an extensive circulation of lymphocytes into the area where the antigen is localized and a proportion of these cells are in some manner stimulated by the antigen. Whether the macrophages in this region of the node process the antigen first or merely capture and possibly retain it on their surface to facilitate contact between lymphoctyes and antigen has not been settled (Fishman and Adler, 1963; Unanue and Cerottini, 1970). In any event it would appear that a general consequence of these circumstances is the

production of a germinal centre and stimulation of the lymphocytes to transform and divide. There is evidence that germinal centres form "de novo" at least during a secondary response to antigenic challenge (Cottier, Keiser, Odartchenko, Hess and Stoner, 1967) and since both active cell proliferation and the presence of specific antibody-forming cells are prominent features of germinal centres it seems likely that these structures are the first sites where antibody production occurs in the lymph node. The route whereby cells leave the germinal centres is not known, but once stimulated, cells probably migrate from the germinal centres and the cortex via the lymph sinuses into the medullary cords of the node.

During the inductive phase of the immune response a specific role for the macrophage has been postulated in processing the antigen. The end result of this process was thought to be a complex of RNA and protein which was passed to the lymphoid cells as a form of antigenic message which initiated transformation and antibody synthesis in these cells (Fishman and Adler, 1963). Subsequently, the presence of antigen was demonstrated in similar RNA preparations, raising questions about the role of RNA in the process (Friedman, Stavitsky and Solomon, 1965; Askonas and Rhodes, 1965). More recently, Roelants and Goodman (1969) have studied the ability of several antigens to form complexes with RNA derived from peritoneal exudate cells. They found that the formation of RNA complexes was unrelated to the potency of the antigen, that it was not an enzyme-dependent reaction, did not require the synthesis of RNA, and was not specific for macrophages. These results suggest that it is unlikely that macrophage derived RNA-antigen complexes play a physiologically significant role in immune induction. Although the role of the macrophage may still prove to be crucial in the initial phases of the immune response it seems at this stage that the primary function of phagocytic cells is in the elimination of antigen and antigen-antibody complexes by phagocytosis and their subsequent catabolism by hydrolytic, lysosomal enzymes.

Cell co-operation of another nature has recently been hypothesized as being relevant to the induction of an immune response. Davies, Leuchars, Wallis, Marchant and Elliott (1967) have shown that thymus-derived lymphocytes undergo mitosis in response to antigen but they do not form antibody. Claman, Chaperon and Triplett (1966) showed that while neither thymus cell suspensions nor bone-marrow cells alone could initiate an antibody response in lethally irradiated animals a mixture of these two types of cells, when injected together, led to the production of antibody. These results suggested some sort of collaboration between thymus-derived and bone-marrow derived lymphocytes in the initiation of the immune response. Miller and Mitchell (1968) and Mitchell and Miller (1968) have also concluded that thymus-derived cells recognize and respond to antigen and that these cells subsequently influence bone-marrow cells to produce the specific antibody. It will be important to have these results substantiated in other systems since it was only possible to demonstrate this phenomenon in a strain of mice which cannot respond normally to a specific antigen after neonatal thymectomy. Furthermore, the magnitude of the immune response obtained by combining thymus and bone marrow cells was much lower than was obtained by using thoracic duct cells in the same experimental model system (Nossal, 1969). Future experiments should show whether the phenomenon is of general relevance to other immune responses.

Regardless of the events in the inductive phase of the response, once antibody-forming cells reach the medulla of the lymph node many of the cells face the prospect of continuing their existence fixed within the tissues that make up the medullary cords while many other cells may leave the lymph node of origin and continue their existence, for a short time at least, as free-floating cells in the lymph stream. The role of these migratory cells which both enter and leave the antigenically challenged lymph node will be considered in more detail.

Changes in Lymph Following Antigenic Challenge

Most of the studies on immune responses occurring in fixed lymphoid tissue have been done by studying histological sections or suspensions of lymph node cells. Relationships between cell proliferation, transformation and the appearance of antibody in cells and in the blood have been studied using both lymph nodes and spleens (Fagraeus, 1948; Leduc, Coons, and Connolly, 1955; Jerne, Nordin, and Henry, 1963; Dutton and Mishell, 1967; Leduc, Avrameas and Bouteille, 1968). Comparatively few studies have been done on the more dynamic aspects of an immune response, taking into account the cells which enter and leave the stimulated tissue.

Ehrich and Harris (1942) did some experiments which purported to show that lymphocytes produced antibody. They made acute collections of lymph from the popliteal lymph nodes of anaesthetized rabbits after injecting antigens into the animal's footpads. Antibody was titrated both in lymph plasma and in lymph cell extracts and they claimed that, as all the cells in the efferent lymph were lymphocytes, antibody synthesis occurred in these cells. Antibody-forming cells have been found in thoracic duct lymph of small animals after immunization but the number of antibody-forming cells detected has been quite small and overshadowed by the large number of cells that appear not to be involved in the particular immune response. Hummeler, Harris, Tomassini, Hechtel and Farber (1966) were able to detect 9 - 33 cells per million making antibody to sheep red cells in rabbit thoracic duct lymph. This was following the injection of antigen directly into mesenteric lymph nodes. They were able to detect 20 - 48 antibody-forming cells per million in popliteal lymph after stimulation in the foot pad. Haskill, Legge and Shortman (1969) were able to detect up to a maximum of 600 haemolytic, plaque-forming cells per million cells in the thoracic duct of rats after immunization.

None of these results appear at first hand to suggest that any large scale migration of antibody-forming cells takes place from a stimulated lymph node by way of the lymph

stream. However, the severe technical deficiencies of all these experimental preparations precluded any quantitative assessment of the significance of cell migration as a physiological consequence of the immune response.

Studies on the popliteal lymph nodes of sheep, utilizing conscious animals with indwelling lymphatic fistulae have provided a completely different picture of the immune response, emphasizing the role of cell migration and the extent to which lymph-borne cells participate in antibody synthesis. The events that occur in the lymph following antigenic stimulation of the popliteal node follow a rapid sequence over a period of several days.

The injection of the antigen into the lower leg may result in changes in vascular permeability and some degree of inflammation at or near the site of injection. Whilst at this stage the production of antibody may not be involved in the reaction this inflammation can still be considered as part of the immune response. The cell content of afferent lymph may change considerably at this time as polymorphonuclear cells leave the circulation and enter the tissues and the lymph. The rate of lymph flow may also increase considerably as the initial reactions to the antigen involve damage to the blood capillaries and the formation of oedema. These initial reactions continue over the first 24-48 hours and subsequently merge into the cellular events that give rise to the formation of specific antibody (Smith, Pedersen and Morris, 1970).

The antigen is taken up by the regional lymphatics and transported to the node in the afferent lymph. If the antigen is a soluble protein most of it will pass through the lymph node; if the antigen is particulate in nature, it will be almost completely extracted from the lymph by macrophages in the node. In addition a good deal of antigen may be phagocytosed by tissue macrophages and monocytes and carried in the afferent lymph by these cells to the regional node (Morris, Moreno and Bessis, 1968; Smith, McIntosh and Morris, 1970). Neutrophils and eosinophils may also take up antigen and assist in transporting it to the node. Whether

the initial inflammatory episode affects the subsequent immune response in terms of the quantity or type of antibody produced has not been determined but, since no inflammatory response can be detected in some situations and yet the subsequent immune response qualitatively appears to be the same, it seems probable that these inflammatory events are not an obligatory prerequisite to an immune response.

The injection of an antigen usually results in a transitory fall in output of cells from the node in the efferent lymph (Hall and Morris, 1965b). The extent to which this occurs varies with the quantity and the type of antigen. Soluble protein antigens usually have very little effect but 100µg of influenza virus will eliminate practically all cells from the efferent lymph for a period of 12 hours or more (Smith and Morris, 1970). The mechanisms responsible for this phenomenon are not known but they may have some relationship with inflammatory changes within the lymph node that occur during the inductive phase of the immune response.

The next phase of the reaction probably involves the interaction of lymphocytes in the lymph node with the antigen. At this time however, the traffic of lymphocytes through the lymph node increases considerably as more and more cells pass from the blood into the mode. This re-circulation is specific for lymphocytes and none of the other types of blood cells appear in the efferent lymph. There is no evidence to suggest that the lymphocytes which appear in the lymph at this stage of the response have been affected in any other way by the antigen.

Between the second and the third day after challenge transformed, basophilic cells begin to appear in the efferent lymph. Many of these cells show signs of ultrastructural change, many will incorporate ^{3}H-thymidine and some have been shown to contain and to secrete antibody (Hall and Morris, 1963; Cunningham, Smith and Mercer, 1966; Hall, Morris, Moreno and Bessis, 1967).

It has been shown that the cells which leave the lymph node via the lymphatic system are important in the

establishment of immunological memory at sites remote from the originally stimulated node (Smith, Cunningham, Lafferty and Morris, 1970). In addition the cells leaving the node or possibly antigen associated with the cells have been shown to stimulate other lymph nodes, leading to the production of amounts of antibody considerably in excess of that formed in the node in which the antigen was initially localized. It has also been shown that when antigen is administered to a node via an afferent popliteal lymphatic the immune response can be confined to the popliteal lymph node providing the efferent lymph from the node together with its cells is drained from the animal. In such a situation the animal develops no systemic immunity and the single lymph node alone becomes sensitized to the antigen (Hall, Morris, Moreno and Bessis, 1967; Smith, Cunningham, Lafferty and Morris, 1970).

The immunological events that occur during the rejection of skin homografts and renal homografts can also be monitored in the draining lymph (Hall, 1967; Smith, Pedersen and Morris, 1970; Pedersen and Morris, 1970) and the pattern of cellular events has been shown to be similar in all these situations to those occurring with a variety of antigens. While the magnitude of the cellular response and the time at which the blast cells reach their peak output can be altered by selecting different antigens and by varying the conditions under which the challenge is made, the overall manifestation of the immune reaction is largely the same in all these different responses.

This thesis concerns an investigation into the natural history of the immune response as it occurs under physiological conditions. The opportunity has been exploited to determine the sequence of cellular and biochemical events that occur in an individual lymph node and in the lymph following an antigenic challenge and a study has been made of the capabilities and life-history of certain members of the cell population that appear in the lymph as a result of the processes of cell proliferation and transformation that are such an important aspect of the immune response in mammals. Whilst it can be stated that most immune responses in

mammals involve the transport of antigen to fixed lymphoid tissue, almost always via the lymphatic system rather than via the blood stream, important exceptions may exist. In some naturally occurring pathological situations the antigen may be fixed within tissues such as with neoplastic tumours or granulomatous reactions to foreign antigens or in conditions brought about by organ transplantation. Immune reactions in these instances may be generated by the migratory component of lymphoid tissue dispatched to the site where the antigen is localized. In other situations such as hypersensitivity reactions, migratory cells rapidly appear at the injection site, even before the antigen has reached the fixed lymphoid tissue. These types of immunological reactions which inevitably involve the lymphatic system at the levels of the afferent lymph, the regional lymph node and the efferent lymph have been included in this present investigation to highlight the variability of the immune response as it occurs in these different circumstances.

CHAPTER II

MATERIALS AND METHODS

Experimental Animals

Sheep

Randomly bred Merino or Merino-Corriedale ewes 3-5 years old were used for all experiments.

Rabbits

The rabbits used were randomly bred from the animal colony of the John Curtin School of Medical Research.

Chickens

Outbred hens from the animal colony of the John Curtin School of Medical Research were used as a source of blood.

Chemicals, Buffers and Solutions

All chemicals were of the Analytical Reagent grade unless otherwise stated.

Sodium Chloride Solution

A 0.9 per cent solution of NaCl was prepared in distilled water; hereafter this solution is referred to as saline.

Phosphate Buffered Saline (PBS)

The following three solutions were autoclaved separately and mixed before use:

I NaCl 8.0 g, KCl 0.2 g, Na_2HPO_4 1.15 g, KH_2PO_4 0.2 g, deionized distilled water (DDW) 800 ml.

II $CaCl_2$ 0.1 g, DDW 100 ml.

III $MgCl_2 \cdot 6H_2O$ 0.1 g, DDW 100 ml.

Calcium, Magnesium Saline

A solution was prepared by dissolving 9.0 g NaCl, 0.028 g $CaCl_2$ and 0.079 g $MgCl_2$ in 1 litre of DDW.

Alsever's Solution (modified)

8 g of sodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$), 4.2 g NaCl, 20.5 g glucose and 8 ml of 10 per cent citric acid were added to 1 litre of DDW. This solution was used for the collection of blood in a ratio of 8 volumes of whole blood to 2 volumes of Alsever's solution.

Hanks' Balanced Salt Solution (BSS)

The following chemicals were dissolved in 1 litre of DDW: NaCl 8.0 g, KCl 0.4 g, $MgSO_4 \cdot 7H_2O$ 0.1 g, $MgCl_2 6H_2O$ 0.14 g, Na_2HPO_4 0.069 g, KH_2PO_4 0.06 g and glucose 1.0 g.

Phenol red (5 ml of a 0.4 per cent aqueous solution/l) was added as a pH indicator.

Unbuffered Balanced Salt Solution (for albumin density gradients)

The following isotonic solutions in DDW were combined: 0.147 M NaCl 121 volumes, 0.147 M KCl 4 volumes, 0.098 M $CaCl_2$ 3 volumes, 0.147 M KH_2PO_4 1 volume, 0.147 M $MgSO_4$ 1 volume.

Tyrode's Solution (for electron-microscopy)

0.40 g NaCl, 0.10 g Na_2HPO_4, 0.02 g KCl, 0.005 g NaH_2PO_4, 0.01 g $CaCl_2$, 0.01 g $MgCl_2$ and 0.10 g dextrose were dissolved in 100 ml of distilled water.

Eagle's Medium

Eagle's medium (Eagle, 1959) was prepared by combining the stock concentrate with a glutamine, $NaHCO_3$ and antibiotic solution in DDW as supplied by the Department of Microbiology, John Curtin School of Medical Research.

Tris Buffer 0.15 M (for Sephadex chromatography)

68.45 g of NaCl and 21.80 g of Tris (Sigma) were dissolved in 3 litres of distilled water. HCl (approximately 110 ml, 1 N) was added to bring the pH to 8.0 at room temperature and the volume was made up to 9 litres with distilled water.

Phosphate Buffer (for gamma-globulin precipitation)

Approximately 9 volumes of 0.1 M Na_2HPO_4 was combined with 1 volume of 0.1 M NaH_2PO_4. The final addition of NaH_2PO_4 was made to bring the pH to 8.0.

Acetate Buffer (for autoradiography)

10.5 ml of 0.2 M acetic acid was added to 39.5 ml of 0.2 M sodium acetate and made up to 100 ml with distilled water resulting in a final pH of 5.2.

Tris - HCl Buffer, 0.05M (for detection of antibody against horse-radish peroxidase)

50 ml of 0.05 M HCl was added to 62 ml of 0.05 M Tris (Sigma). The final pH was 7.5.

Phosphate Buffer, 0.1 M (for detection of antibody against horse-radish peroxidase)

36 ml of $Na_2HPO_4 \cdot 2H_2O$ (17.81 g/l) was added to 14 ml of NaH_2PO_4 (13.80g/l). The final pH was 7.3.

Acetate Buffer, 0.1 M (for β-glucuronidase assay)

a - 0.1 M sodium acetate$\cdot 3H_2O$ (13.61 g/l distilled water)

b - 0.1 M glacial acetic acid (5.8 ml made up to 1000 ml with distilled water)

for pH 4.6, 49 ml of a + 51 ml of b

for pH 5.0, 70 ml of a + 30 ml of b

Glycine Buffer, pH 11.2 (for β-glucuronidase assay)

4.10 g of glycine and 3.16 g of NaCl were dissolved in 100 ml of distilled water. 3.0 ml of concentrated NaOH (1 g of NaOH/1.0 ml of H_2O) was added and the volume made up to 200 ml with distilled water. The pH was adjusted to 11.2 with a few drops of concentrated HCl and the final volume made up to 250 ml with distilled water.

Citrate Buffer, 0.05M (for acid phosphatase assay)

0.410 g of citric acid and 1.125 g of sodium citrate were disolved in 100 ml of distilled water. The final pH was 5.0.

Citrate Buffer, 0.1M (for N-acetyl-β-D-glucosaminidase assay)

a - 0.1M sodium citrate (dihydrate) 29.41 g/l

b - 0.1M citric acid (monohydrate) 21.01 g/l

For pH 5.0, 65.0 ml of a was added to 35.0 ml of b

Glycine Buffer, pH 10.7 (for N-acetyl-β-D-glucosaminidase assay)

Glycine (0.133M), 9.98g/l; NaCl (0.067M), 3.92 g/l and Na_2CO_3 (0.083M), 8.80 g/l were combined. The reagents were dissolved in 450 ml of distilled water, the pH was adjusted to 10.7 with approximately 45 ml of N/1 sodium hydroxide and finally made up to 1000 ml with distilled water.

Enzyme Substrates

Either British Drug Houses or Sigma products were used.

p-Nitrophenyl phosphate (for acid phosphatase) · 82.5 mg was dissolved in 5.0 ml of citrate buffer (5.5×10^{-3}M). This solution was stable for one week when stored at 4°C.

Phenolphthalein β-glucuronide (for β-glucuronidase) · 25.0 mg was dissolved in 5.0 ml of acetate buffer (0.01M) and stored at 4°C for periods up to 1 month.

Phosphate-pyruvate solution (for lactic dehydrogenase) · 700 mg of K_2HPO_4, 90 mg of KH_2PO_4 and 3.0 mg of sodium pyruvate were dissolved in 80 ml of double distilled water. This solution was stable for several months when stored at 4°C.

p-Nitrophenyl-N-acetyl-D-glucosaminide (8mM, for N-acetyl-β-D-glucosaminidase assay) · 13.69 mg was dissolved in 5 ml of 0.1M citrate buffer.

Reduced Nicotinamide Adenine Dinucleotide (for lactic dehydrogenase)

10 mg of NADH-Na_2 was dissolved in 1.5 ml of phosphate-pyruvate solution. This solution must be stored at 4°C and kept for only 1 week.

Mercapto-ethanol Solution

A 0.2 M solution of 2-mercapto-ethanol (Eastman Organic Chemicals) in saline was prepared fresh before use.

Leishman Stain

0.15 g of Leishman stain (British Drug Houses, Ltd.) was dissolved in 100 ml of methanol.

Azure Stain

A 0.1 per cent Azure A solution was prepared in acetate buffer, pH 5.2.

Fluorescent Stains

Anti-serum conjugated with fluorescein isothiocyanate against sheep gamma-globulin and rabbit gamma-globulin (Microbiological Associates, Inc., Bethesda, Maryland) was used.

Complement

Lyophilized guinea-pig serum (Commonwealth Serum Laboratories, Melbourne) was reconstituted with saline and used fresh. On some occasions fresh sheep lymph was used as a source of complement.

Radio-isotopes

Both isotopes were from the Radiochemical Centre, Amersham.

Thymidine (methyl tritiated) · The stock solution had a specific activity of 5 Ci/mM. This was diluted as indicated in the text.

Uridine (5-tritiated) · The stock solution had a specific activity of 5 Ci/mM. This was diluted as indicated in the text.

T-1824 Solution

250 mg of T-1824 dye was dissolved in 100 ml of saline and autoclaved.

Trypan Blue Solution

A 2.0 per cent solution of Trypan blue was made in PBS.

Biuret Reagent

9.0 g sodium potassium tartrate was dissolved in 400 ml 0.2 N NaOH (carbonate-free). 3.0 g of finely powdered $CuSO_4 \cdot 5H_2O$ was added, followed by 5.0 g of KI. The final volume was made up to 1 litre with carbonate-free 0.2 N NaOH.

Scintillation Medium

Ten ml of a toluene medium containing 4 g/l of PPO (2,5-phenyloxazole) and 0.2 g/l of POPOP (1:4-di 2-(5-phenyloxazolyl) benzene) was used with glass fibre discs.

An alternative medium was also used containing 1 volume of Triton X-100, 2 volumes of toluene and 0.2 g/l of PPO.

Anaesthetics

Thiopentone sodium, B.P. ('Intraval' Sodium, May & Baker, Ltd.) as a 5 per cent solution in distilled water was used for the induction of anaesthesia.

Halothane, B.P. ('Fluothane', I.C.I., Ltd.) was used for maintenance of anaesthesia in a closed circuit with a Boyle apparatus.

Pentobarbitone sodium, B.P. ('Nembutal', Abbot Laboratories Pty. Ltd.) was used for some anaesthetic procedures and for euthanasia.

Antibiotics

Penicillin ('Crystapen', Glaxo-Allenburys Aust. Pty. Ltd.) was used to dust surgical wounds and to control bacterial growth in lymph samples.

Streptomycin-penicillin ('Streptopen', Glaxo-Allenburys) containing 250,000 U of procaine penicillin and 0.25 g of dihydrostreptomycin per ml was used for injection purposes.

Neomycin. Neomycin sulphate (Penick and Co., New York) containing 737 µg of neomycin base/mg was used in the lymph collection bottles.

Streptomycin. Streptomycin sulphate (Glaxo-Allenburys) was used in the lymph collection bottles.

General Methods

Collection of lymph and blood samples

Lymph was collected into plastic collection bottles containing heparin and antibiotics. Two ml of a solution containing 500 units of heparin (Pularin, Evans), 500 units of penicillin (Crystapen), 50 µg of streptomycin and 50 µg of neomycin were normally used or, alternatively a small amount of the dry mixed powders was added to the bottles. The period over which the lymph was collected varied depending on the experiment. When it was necessary, sterile collection bottles were used.

Samples of sheep blood were obtained from the jugular vein with a hypodermic syringe that contained either heparin or Alsever's solution in the appropriate amount. Blood samples were taken from the wing vein of chickens and from the heart or ear vein of rabbits. Where necessary, whole blood was allowed to clot and the serum complement inactivated by heating to 56°C for 30 minutes. Samples of serum were stored frozen at -40°C.

Cell Counts

A model B Coulter Counter (Coulter Electronics, Hialeah, Florida) with a 100μ aperture tube was used for all cell counts. The machine was calibrated with polystyrene particles of known dimensions and the readings were done using an aperture setting of 1 and current setting of 1/2. With the lower threshold set at 10.1 and the upper threshold set at 42.5, approximately 90 per cent of normal sheep efferent lymphocytes were counted. With the lower threshold at 42.5 and the upper threshold disabled, the remainder or the "large" cells were counted. During an immune response the percentage of large cells increased up to 50 per cent in some instances and this provided a reasonable correlation with the number of basophilic cells identified in cell smears. The percentage of large cells was higher in afferent lymph due mainly to the presence of macrophages. Polymorphonuclear cells had a characteristic size distribution slightly larger than normal lymphocytes and these could be recognized on the oscilloscope when their numbers were high. In most situations, differential counts, however, were made on Leishman stained cell smears. Red cells which were occasionally found in lymph were not counted using these settings.

Cell Smears

The cell pellet from centrifuged lymph was resuspended in a volume of sheep serum approximately equal to the size of the pellet, smeared on glass slides, air dried and stained. One ml of Leishman stain was poured onto the slide and left for 5 minutes; after this time the stain was diluted by the

addition of 2.5 ml of distilled water. The slides were left a further 5 minutes, rinsed with water, air dried and examined under an oil immersion lens. Alternatively cell spreads were prepared by using a cytocentrifuge as described by Dore and Balfour, (1965). This had the advantages that the cells were more evenly distributed and also only a million cells were required to make a spread.

For autoradiographs the smears were prepared in the same manner, air dried and fixed in methanol for 10 minutes.

For fluorescent antibody staining the cell smears were air dried and fixed in acetone for 10 minutes.

For demonstrating the presence of antibody against horse-radish peroxidase, cell smears were fixed in ethanol-ether (60:40).

Cells were classified in the following categories according to their morphology and staining characteristics:

1) normal lymphocytes

These cells included small, medium and large sizes (5-15μ in diameter) with a small rim of pale staining cytoplasm.

2) basophilic cells

These cells were characterized by cytoplasm which stained intensely basophilic with Leishman stain. Their overall size was usually larger than the mean size of the lymphocytes. These cells made up less than 1 per cent of the normal cell population of lymph but their numbers increased during the immune response. These cells are also referred to as blast cells, antigen-stimulated cells and transformed cells. Plasma cells and Mott or Marshalko cells (vesicle-containing) were included in this class. Details of their morphological characteristics appear in the text.

3) macrophages

Mononuclear cells of large size with ruffled cytoplasm which usually contained granular material were classed as macrophages. These cells were rare in efferent lymph but comprised up to 20 per cent of the afferent lymph cell

population. The cytoplasm of these cells was usually slightly basophilic.

4) neutrophils

Polymorphonuclear cells were similar to those of other mammalian species. Their cytoplasmic granules stained palely.

5) eosinophils

These cells were also typical of other mammalian blood eosinophils with lobulated nuclei and red staining cytoplasmic granules.

Differential counts were done on random fields of the smears counting a total of about 500 cells.

Cell Cultures

Lymph cells that had been collected in sterile bottles were washed twice with Hanks' BSS and incubated at 37°C in Eagle's medium containing 10 per cent foetal lamb serum or other proteins as described in the text. The culture bottles contained 3 ml of cell suspension at a concentration of 3×10^6 cells/ml. The bottles had loose fitting caps, and a mixture of 5 per cent CO_2 in air was maintained in the incubator.

Preparation of Cells and Tissues for Electron-microscopy

Lymph samples were spun down in conical centrifuge tubes and the supernatants decanted. The cell pellets ($1\text{-}5 \times 10^7$ cells) were fixed in 1.25 per cent glutaraldehyde (Fluka AG, Switzerland) in Tyrode's solution for 10 minutes in an ice bath. This solution was pipetted off and a 1 per cent osmium tetroxide solution in Tyrode's was added and left for 2 hours. The pellet was broken up into several pieces during this fixation. The osmium solution was pipetted off and the fragments were washed once with Tyrode's solution. A 1.0 per cent solution of formaldehyde in Tyrode's was added to the centrifuge tube for 30 minutes at room temperature. This was then replaced with a 1.0 per cent aqueous solution of uranyl acetate and left for 2 hours. Dehydration was carried out using successive changes

of 50, 70, 90 and 100 per cent acetone. The 100 per cent acetone was stored over $CuSO_4$ and the final dehydration step was repeated twice. Infiltration with resin (Durcupan, Fluka AG, Switzerland) was carried out over a period of 3 hours by gradually increasing the ratio of resin to acetone. A fresh preparation of resin was used for embedding and the blocks were kept at 45°C for two days.

Samples of tissue were handled in the same way except they were cut into small pieces in 1.25 per cent glutaraldehyde immediately upon their removal from the animal.

Sections were cut on an LKB Ultramicrotome using glass or diamond knives and these were mounted on uncoated copper grids. The sections were stained with Millonig's lead stain (Millonig, 1961) and examined with a Philips EMU200 electron microscope or a Siemens Elmiskop I.

Preparation of Autoradiographs

Light-microscopy · Cells were suspended in Eagle's medium which contained 10 per cent calf serum or isologous lymph. Tritiated thymidine, 1 μCi/ml, was added and the cells incubated at 37°C for periods up to 1 hour. The cells were then centrifuged down and washed 3 times with cold Eagle's medium. Methanol-fixed smears were covered with Kodak AR 10 Stripping Film. Slides were developed with Kodak D19 developer at various times after they were covered with film so as to obtain the desired grain density. After acid fixing and drying, slides were stained with 0.1 per cent Azure A for 15 minutes, washed in water, dried and examined.

Electron-microscopy · Electron microscope autoradiography was done using the method described by Salpeter and Bachmann (1964). Ribbons of ultrathin sections were placed on glass slides coated previously with a 0.5 or 1.5 per cent solution of collodion in amyl acetate. A layer of carbon approximately 100 Å thick was then deposited over the sections and the slides dipped in a solution of Ilford L4

emulsion diluted about 1 in 5 with double distilled water so as to give a layer showing a purple interference colour. The slides were thoroughly dried, stored for 4 weeks at 4°C and then developed for 2 1/2 or 3 minutes with Microdol X (Kodak) developer at 23°C. After development the collodion film was floated off the slide onto a water bath and the section picked up on 300 mesh grids. The grids were treated with amyl acetate for 5 to 15 minutes to dissolve some of the collodion; this increased the contrast of the sections in the electron beam.

Histology

Tissues were fixed in formol-saline and paraffin sections were prepared by Mr. R. Hill of the John Curtin School of Medical Research. Routine material was stained with haematoxylin and eosin or pyronin-methyl green.

Cell Viability

A cell suspension in serum-free medium was added to an equal volume of 2 per cent trypan blue solution and the number of cells taking up the dye after a period of 3-5 minutes was determined using a haemocytometer.

Preparation of Antisera

Rabbit anti-sheep serum · Rabbits were given a primary injection of complete Freund's adjuvant containing an equal volume of emulsified sheep lymph plasma. Two subcutaneous injection sites were used immediately posterior to the scapulae. Two or three intravenous injections of 1 ml of lymph plasma were given at bi-weekly intervals. An estimate of the titre of the anti-serum was made by using the ring test consisting of 0.5 ml of lymph plasma layered on top of the rabbit serum. The precipitation reaction was observed after 30 minutes at 37°C.

Rabbit anti-sheep lymphocyte serum · Efferent sheep lymphocytes (2×10^8 cells) were emulsified in complete Freund's adjuvant and injected subcutaneously

into rabbits following the procedure described in the preceding paragraph. Second, third and fourth injections of 2×10^8 cells were given intravenously without adjuvant. Samples of blood were taken from the rabbit and the agglutinating and cytotoxic capacity of the sera were determined.

Gamma-globulin Precipitation, Separation and Identification

The mixed globulins were precipitated from serum by the addition of 18 g of anhydrous sodium sulphate per 100 ml of serum. The precipitated proteins were dissolved in phosphate buffer and two further precipitations were then carried out with the total sodium sulphate concentration at 12 per cent. The final solution of gamma-globulins was dialyzed against PBS.

The separation of IgM and IgG was done using a 4 x 100 cm column packed with Sephadex G-200. An LKB ReCyChrom apparatus was used which infused Tris buffer through the column at a constant rate. A 5-10 ml sample of lymph or blood plasma or the gamma-globulin solution was added to the column and approximately 10 ml fractions were collected on a time basis. An automatic protein scan was recorded on a chart. The apparatus was run in a $4^{\circ}C$ room.

The reduction of IgM by 2-mercapto-ethanol was carried out by incubating an equal volume of lymph or a gamma-globulin solution in 0.2 M 2-mercapto-ethanol solution at $37^{\circ}C$ for 30 minutes prior to the titration of antibody.

Protein Determinations

Estimation of protein was carried out by using the Biuret reaction similar to the procedure outlined by Kabat and Mayer (1961). For lymph samples 0.1 ml was mixed with 0.9 ml of saline. 1.5 ml of Biuret reagent was then added, mixed, left for 30 minutes at room temperature and read at 555 nm in a Gilford Model 300-N micro-sample spectrophotometer against a blank containing only Biuret reagent. For albumin determinations, either

0.1 or 0.2 ml of lymph was made up to 1 ml with saturated sodium sulphate solution and the globulins centrifuged out. 0.5 ml of the supernatant was then made up to 1 ml with saline, 1.5 ml of Biuret reagent added, mixed and measured as in the case of total protein determinations. A standard curve was prepared (Figure II-1a) by dissolving known weights of bovine serum albumin fraction V (Armour, Chicago) in distilled water. The protein concentration of lymph in g/100 ml was given by 10 x optical density x the regression coefficient from the standard curve.

Scintillation Counting

Cells were centrifuged out of the radioactive medium and washed twice with Hanks' BSS. A drop of protein carrier (2 per cent BSA in saline) was added to the cell pellet followed by 5 ml of cold 5 per cent trichloracetic acid. The cells were centrifuged and washed once with cold 5 per cent trichloracetic acid. The precipitate was dissolved in 1 ml of formic acid and transferred to a scintillation vial which was then filled with 15 ml of Triton-toluene scintillator. Counting was carried out using a Beckman LS-100 liquid scintillation counter and enough counts were allowed to accumulate to reduce the error to approximately 2 per cent. None of the samples contained red cells so that corrections for quenching by haemoglobin were not necessary.

An alternative procedure was based on a method used for cell cultures (Wilson, Blyth and Nowell, 1968). The washing and precipitation of nucleoproteins was the same but the final precipitate was dissolved in 0.5 ml of 2 M NH_4OH. 0.1 ml volumes were then transferred to Whatman glass fibre discs (W. & R. Balston, Ltd.) and dried at 110°C for 20 minutes. The dried discs were placed in scintillation vials and 10 ml of toluene scintillator was added.

Sterilization

Solutions were either autoclaved or Seitz filtered. Smaller volumes of sera and solutions were filtered through 0.45 μ 'Millipore' filters.

Dialysis

Solutions were dialyzed either in cellulose dialysis tubing bags using at least 10 volumes of dialysis medium to 1 volume of solution in the bag or alternatively, in a 50 ml capacity 'Diaflo' apparatus. Dialysis was done at 4°C.

Statistical Methods

The mean "$\bar{x}$" of a series of observations x1, x2, x3 ....x_n is given by $\bar{x} = \frac{\Sigma x}{n}$ where "n" is the number of observations and x is the sum of the observations.

The variance "V" is taken as the total sum of the squared deviations divided by one less than the total number of observations and is given by $V = \frac{\Sigma (x-\bar{x})^2}{n-1}$.

The standard deviation "S.D." is calculated as the square root of the variance.

$$S.D. = \sqrt{\frac{\Sigma (x-\bar{x})^2}{n-1}}.$$

The significance of the difference between two means is calculated using Student's 't' test and is given by

$$t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\frac{N_1 + N_2}{N_1 N_2 (N_1 + N_2 - 2)} \left[\Sigma (x-\bar{x}_1)^2 + \Sigma (x-\bar{x}_2)^2 \right]}},$$

where N_1 and N_2 represent the number of observations in each group.

Detailed Methods

Surgical Methods

Animals were starved for 24 hours prior to surgery. For the induction of anaesthesia, thiopentone sodium was administered (0.1 g per 5.0 Kg body weight) intravenously. A cuffed McGill endotracheal tube was used for intubation

and anaesthesia was maintained with halothane and oxygen using the closed circuit of a Boyle's anaesthetic machine (British Oxygen Company). For procedures of short duration, intravenously administered pentobarbitone sodium was used at a dose rate of 12.5 mg per Kg. Small additional doses were given when required, to sustain anaesthesia.

Cannulae for lymphatic ducts were either polyvinyl or polyethylene (Dural Plastics, New South Wales). The operative area of the animal was prepared by clipping the wool as close to the skin as possible and then scrubbing the area with a 1.0 per cent solution of chlorhexidine ('Hibitane', I.C.I.). The animal was covered with sterile drapes. The usual sterile operating precautions were taken throughout the surgery.

Cannulation of the efferent popliteal lymphatics. The technique used was that described by Hall and Morris (1962). An incision was made through the skin and subcutaneous tissues from a point 2-3 cm below the sciatic tuberosity to a point about 10 cm down the leg. Bleeding was controlled by electro-cautery. The semitendinosus muscles and the biceps femoris were separated with wound retractors and the popliteal fossa exposed. Muscular branches of the posterior femoral artery and vein were tied with two ligatures of 0 silk and then cut. The posterior femoral vein was then exposed by blunt dissection and the efferent lymphatic duct found. Its usual position is medial and adjacent to the femoral vein. A ligature of 3/0 silk was tied around the duct as far from the node as possible. The duct was cleaned of adherent fat and connective tissue and a second ligature loosely applied about 1 cm below the occluding tie. An appropriate sized cannula was positioned in the same direction as the duct by leading it through a stab incision made through the skin. A small incision was then made in the lymphatic with iridectomy scissors in a region free of any valves. The end of the cannula was positioned in the duct and secured in place with the lower ligature. One or two other ligatures were then used to firmly fix the cannula in place. The wound was dusted with

powdered penicillin and the skin incision closed with Michel clips. A purse-string suture was used to secure the cannula externally and a plastic bottle holder was sutured to the skin.

Cannulation of the afferent popliteal lymphatic · The afferent popliteal lymphatics can be located accompanying the recurrent tarsal vein immediately above the hock. An incision 5 cm long was made over this vein and the skin and fascia covering the vein retracted. Two to four afferent lymphatics can usually be identified on both sides of the vein. For the collection of afferent lymph, a procedure similar to that described for the efferent duct was followed (Hall and Morris, 1962). In some experiments it was necessary to insert an indwelling cannula into an afferent duct to enable the continuous administration of material to the lymph node. A single duct was isolated and loosely tied with two ties about 2 cm apart. Light tension was applied to these threads and the duct cleaned thoroughly. A third suture was loosely tied between the first two. The duct was cut with iridectomy scissors and the cannula slid up the lymphatic in the direction of lymph flow for about 1 cm and secured with the middle suture. The top suture was then removed, the cannula positioned through a stab wound in the skin and fixed firmly with a further one or two sutures. Approximately 0.5 ml of sterile T-1824 dye solution was infused into the cannula. If the positioning was correct, there was no leakage of dye around the duct and dye appeared in the efferent lymph almost at once. The skin was closed with Michel clips and a 1-2 m coil of cannula which had been flame sealed at the end was secured to the skin to enable the cannula to be connected subsequently to an infusion apparatus.

Cannulation of the efferent prefemoral lymphatic · The drainage area of the prefemoral lymphatics is more diffuse than that of the popliteal area. An efferent prefemoral vessel was only cannulated to obtain unstimulated efferent lymph cells. The procedure has been described (Cole,

1969). A vertical incision (5-10 cm) was made slightly anterior to the cranial border of the tensor fascia lata. The subcutaneous tissues were separated and the efferent lymphatics found lying in association with the blood vessels supplying the prefemoral lymph node. This node can be palpated readily. The cannulation procedure was similar to that described for the popliteal duct. After operation all the animals were kept in metabolism cages (Figure II-2).

Preparation and Administration of Antigens

Chicken erythrocytes · Hens were bled immediately before the cells were required. After centrifugation of the blood, the buffy coat was pipetted off and the erythrocytes washed twice with PBS. One ml of the cell suspension containing 2×10^9 cells was injected.

Swine influenza virus · A sample of viable swine influenza virus (strain Shope 15) was obtained from Dr. Maer-Ewert of the Department of Microbiology, the John Curtin School of Medical Research. Ten-day old fertile chicken eggs were inoculated and the allantoic fluid was harvested 4 days later. Particulate debris was centrifuged out and to the supernatant was added one-tenth its volume of packed, washed chicken erythrocytes. The virus was adsorbed onto the cells at $0^{\circ}C$ for 30 minutes. The cells were centrifuged out, resuspended in 20 ml of saline and incubated at $37^{\circ}C$ for 1 hr to elute the virus off the cells. The supernatant was removed and stored at $4^{\circ}C$. One ml of virus suspension (10 to 20,000 haemagglutinating units) was injected.

Serum antigens · Rabbit serum or anti-serum was sterilized and injected in volumes of 2 ml or less.

Salmonella muenchen · Salmonella muenchen cultures were maintained on nutrient agar slopes at $4^{\circ}C$ and fresh tubes were inoculated at monthly intervals. Petri dishes containing nutrient agar were inoculated and left at $37^{\circ}C$ for 1-2 days until a continuous lawn of organisms had grown. These organisms were washed from

the surface of the agar using small volumes of saline and the washings boiled for 30 minutes. The concentration of organisms was determined by comparison with opacity tubes ('Wellcome') containing a standard number of Salmonella organisms. A stock solution containing 2×10^9 bacteria/ml was kept at $4^{\circ}C$ in 5 ml bottles. For injection purposes 0.5 ml was used in all cases.

Salmonella muenchen lipopolysaccharide was prepared by the method of Halliday and Webb (1965). Two 1 litre sterile flasks containing nutrient broth (Difco) were inoculated with organisms. The broth cultures were maintained at $37^{\circ}C$ on a mechanical shaker for 24 hours and then centrifuged at 6,000 rpm for 30 minutes to deposit the bacteria. The supernatant was removed and the bacteria washed twice in 400 ml of saline. 16 ml of 1 N NaOH was added to the bacterial suspension and the mixture incubated at $37^{\circ}C$ for 16 hours. The suspension was neutralized with 1 N HCl after which it was centrifuged at 6,000 rpm for 30 minutes and the supernatant separated. 0.5 of a volume of acetone was mixed with one volume of the supernatant and centrifuged. A further 0.5 volume of acetone was added and the mixture kept at $4^{\circ}C$ for 3 hours. The precipitated lipopolysaccharide was collected by centrifugation, resuspended in a small volume of acetone and again centrifuged. The acetone was evaporated and the final deposit dried in a desiccator over $CaCl_2$ and then ground to a fine powder.

Red blood cells (either from sheep or from chickens) were coated with the lipopolysaccharide and used as indicator cells either for plaque assays or antibody titrations. Equal volumes of a 5.0 per cent solution of red cells and of a lipopolysaccharide solution containing 50 µg/ml were incubated at $37^{\circ}C$ for 60 minutes. The cells were then centrifuged out and washed twice in saline.

Horse-radish peroxidase · Horse-radish peroxidase (Type I - 'Sigma') was injected in a dose of 10 mg dissolved in saline. For primary immunization and some secondary injections, this was emulsified in 0.5 ml of incomplete Freund's adjuvant (Difco).

Echinococcus granulosus · An antigenic mixture containing suspended protoscolices was obtained from the Department of Zoology, The Australian National University. The protoscolices were obtained from the fluid of hydatid cysts found in sheep viscera and were resuspended in a balanced salt solution. Two ml or less of the mixture was injected.

Homologous lymphocytes · Fresh lymphocytes were obtained from draining efferent lymphatics. The cells were washed twice with PBS, counted and injected in varying doses.

Administration of antigens · In most situations where antigens were injected, they were given subcutaneously in the lateral aspect of the lower leg. Alternatively the antigen was administered via a cannulated afferent lymph vessel. Continuous infusions were begun at least 24 hours after surgery at a rate varying from 0.4-1.0 ml/hour by means of a slow infusion apparatus (Palmer). The continuity of the afferent-efferent connection was tested with a solution of T-1824 dye immediately before the infusion was started.

NLT reactions were induced by injecting homologous lymphocytes intra-dermally in the wool-free skin of the medial aspect of the thigh as described by Jones and Lafferty (1969). A volume of cell suspension (0.1 ml) was injected with a tuberculin syringe and a 27 gauge needle. Skin measurements were made with a pair of 'Schnelltaster' skin calipers before and at daily intervals after injection.

Titration of Antibody

Complement was inactivated by incubating samples of lymph and blood serum at 56°C for 30 minutes. IgM and IgG antibody was distinguished by incubating lymph or plasma samples in an equal volume of 2-mercapto-ethanol solution for 30 minutes at 37°C prior to their titration. The effectiveness of this method in reducing IgM antibody was tested as outlined in the text. In most cases the samples were prepared in doubling dilutions and the titre was expressed in $\log_2$ units. The first tube was designated as 0 and subsequent tubes 1,2,3, etc. The starting dilution was usually 1:2 but this varied with different assays. For example after incubation in 2-mercapto-ethanol, the starting dilution was 1:4. In situations where a comparison was drawn between titres the same starting dilution was used. If this was not possible a correction was made.

Haemagglutination and haemolysin assays · Calcium, magnesium saline was added to Perspex haemagglutination trays and 2-fold dilutions of samples were made using a Takatsky loop. Chicken erythrocytes were used as test cells and end-points were read after 35 minutes. A conventional 50 per cent end-point was determined using a standard description of agglutination patterns. Lytic antibody was detected by adding 0.025 ml of a 1:4 dilution of reconstituted lyophilized guinea-pig serum and the trays were read after standing at 4°C overnight.

Anti-lymphocyte antibody · Using heterologous, anti-sheep lymphocyte antibody, 2-fold dilutions were made as described in the preceding paragraph. Cells from efferent lymph were used as test cells and the trays were left for 2 hours at room temperature before reading. Drop samples from each well were added to microscope slides and evidence of cell aggregation was determined using a microscope. End-points were taken as the last dilution showing cell aggregation. Cytotoxic assays were done using the trypan blue exclusion method.

For the detection of antibody directed against homologous sheep lymphocytes, a more sensitive method was used. This was the capillary migration procedure described by Thompson, Severson, Lavender, Forland and Russe (1968). Serial dilutions were made in agglutination trays in the same manner as before. Test lymphocytes from the donor sheep were washed twice and resuspended at a concentration of 10^7/ml in a medium consisting of 3 parts PBS:1 part foetal calf serum:1 part Alsever's solution. 0.025 ml of this suspension was added to each well. The tray was shaken and left standing for at least 10 minutes. 25 µl capillary tubes were filled from each well and one end was sealed in a flame. The tubes were centrifuged to bring the cells to one end of the tube and these were then taped onto microscope slides and left in an inverted position for 10 minutes. In the control tubes and tubes containing no specific antibody, the lymphocytes fell from the pellet on the bottom and migrated down the tube. When antibody was present, the cells did not migrate. End-points were judged as the last tube which migrated less than the controls when they were compared under the low power objective lens of a microscope. Titres were consistently reproduced ±1 tube. Further comments on the method are given in the text.

When the heterologous ALS was titrated in this manner, the titre recorded was 5 tubes higher than was obtained using the slide agglutination procedure.

The Detection of Intra-cellular Antibody to Horse-radish Peroxidase

Cells producing antibody against horse-radish peroxidase were demonstrated by the histochemical reaction which results in the formation of a dense precipitate when the enzyme reacts with its substrate in the presence of 3, 3'-diaminobenzidine. The method of Leduc, Avrameas and Bouteille (1968) was followed. Cells were centrifuged from the lymph and washed twice with Hanks' solution. For light microscopy, smears were prepared and fixed in methanol-ether for 30

minutes and air-dried. A proportion of the cells was fixed in 1.25 per cent glutaraldehyde for 30 minutes in 0.1 M phosphate buffer and processed as controls for electron microscopy. A second portion of the cells was fixed for 30 minutes in 1.25 per cent glutaraldehyde and together with the cell smears exposed to a solution of 50 µg/ml of horse-radish peroxidase for 30 minutes at room temperature with continual agitation. The cells were then washed twice in 0.1 M phosphate buffer. Smears were treated with a solution of 2-5 mg of 3,3' diaminobenzidine in 10 ml of 0.05 M Tris-HCl buffer containing 0.01 per cent H_2O_2 for 30 minutes at room temperature to reveal the presence of antigen-antibody conjugates. The suspension of lymph cells was refixed for 15 minutes with 1.25 per cent glutaraldehyde, washed twice in 0.1 M phosphate buffer and then treated with the diaminobenzidine-H_2O_2 reagent. The cells were again washed twice with phosphate buffer and fixed further in 1 per cent OsO_4 for 30 minutes and processed for electron microscopy.

Lymph nodes were divided into medulla and cortex immediately after they were removed from the animal. The tissue was cut into fragments and teased apart with dissecting needles. Fragments were separated and the cells dispersed by passing through a stainless steel, 60 mesh sieve. The cells were processed in the same way as the lymph cells. Controls were performed on the lymph cells collected before antigenic stimulation, and no positive lymphoid cells were found in these samples either in light microscope smears or in electron microscope sections. Other controls consisted of incubating the cells in the presence of hydrogen peroxide and diaminobenzidine without previously exposing the cells to horse-radish peroxidase solution. These preparations were invariably negative when carried out on cells collected before antigenic challenge and on cells collected from 24 hours onwards after antigenic challenge. The presence of antigen in samples of lymph collected immediately following the injection of

peroxidase under the skin gave rise to positive reactions on the surface of some cells unless they were washed. These reactions were diffuse and confined to the surface of the cells and easily distinguished from the reaction given by antibody-forming cells. Endogenous peroxidase activity was revealed also by this method in eosinophils and red cells.

Lymph cells collected during an immune response to Salmonella flagella were reacted with horse-radish peroxidase, H_2O_2 and diaminobenzidine as a further control. No positive cells were found. Occasionally some cells in the controls showed a non-specific type of staining in which a reaction product appeared on the cell surface. Most cells showing this reaction appeared to be damaged or poorly fixed.

Plaque Assay for Antibody-forming Cells

Cells producing antibody to chicken erythrocytes or Salmonella lipopolysaccharide were assayed using the Jerne plaque technique (Jerne, Nordin and Henry, 1963) as modified by Cunningham and Szenberg (1968). Chambers were made by applying two strips of 'Scotch' brand double-coated tape No. 4010 22 mm apart to a microscope slide. The backing of the tape was removed and a coverslip 22 mm x 33 mm was applied and pressed to seal onto the tape. This provided a chamber of approximately 0.05 ml volume.

Lymph or lymph node cells were centrifuged, washed twice with cold Eagle's medium and resuspended to give a concentration of 10^5-10^7 cells per ml. Sheep erythrocytes (usually isologous cells) that had been coated with lipopolysaccharide or chicken erythrocytes at a concentration of 1 volume of packed cells to 6 volumes of Eagle's medium were added to the lymphoid cells. To 0.05 ml of lymphoid cells, 0.1 ml of red cells was added together with 0.05 ml of guinea-pig complement from which any antibody to Salmonella lipopolysaccharide had been absorbed. 0.05 ml of this mixture which was

maintained in an ice bath throughout was then warmed to 37°C and added to a slide chamber. The sides of the chamber were sealed with heated paraffin-Vaseline and incubated at 37°C for 30 minutes. The chambers were examined under low magnification and the number of areas of lysis which contained a nucleated cell in the centre, were counted.

The number of plaque-forming cells (PFC) per chamber was converted to the number of PFC per million lymphoid cells by the following calculation: the initial 0.5 ml of lymphoid cells was diluted to a volume of 0.65 ml; therefore the concentration of cells in the reaction mixture = $X \times \frac{0.5}{0.65} \times 10^6$/ml. Since the chamber contained 0.05 ml, then the number of PFC/ml of reaction mixture = 20 x number of PFC/chamber and $PFC/10^6 = \frac{20 \times PFC/chamber}{X \times 0.77}$ or more simply, $PFC/10^6 = \frac{26 \times PFC/chamber}{\text{cell conc./ml} \times 10^6}$. Indirect plaque assays using antiserum in the reaction mixture are described in the text.

Preparation of Albumin Equilibrium-density Gradients

The general method followed was based on that described by Shortman (1968). Crystalline bovine serum albumin, fraction V (Armour and Co., Chicago, Illinois) was dissolved in distilled water (15-20 per cent solution) and dialyzed for 2 days to remove any traces of salt. It was then Seitz-filtered, freeze-dried in sterile Petri dishes and stored in a desiccator. Stock solutions of albumin were made up by dissolving 100 g of albumin in 145 ml of balanced salt solution, 5 ml of water and 0.2 ml of antibiotic solution. The powder was layered on top of the liquid in a sealed container and stirred with a magnetic stirring rod at 4°C for 1-2 days. Solutions of 15 and 25 per cent were further diluted in balanced salt solution to prepare the gradients. 3.5 ml of cold 35 per cent albumin was added to a centrifuge tube containing 5-8 x 10^7 cells in a pellet on the bottom. The cells were mixed into the albumin with a pipette and then added to a chamber containing an electric powered

stirring paddle. An outlet at the bottom of the chamber was separated into two exit channels and led through a peristaltic pump. An inlet channel in the bottom of the chamber contained the 15 per cent albumin solution. By pumping the chamber at twice the rate at which the 15 per cent albumin entered, a near linear gradient was formed and collected in an 8 ml nitro-cellulose tube. A 0.3 ml cushion of 40 per cent albumin was added to the tube before the mixed material was run in.

The gradient was centrifuged at 3800 g for 45 minutes until the cells reached their equilibrium-density level. The refrigeration apparatus of the centrifuge was shut off during the spin to avoid disturbing the gradient, but the temperature was maintained at less than 10°C. After centrifugation, the cells could be seen throughout the gradient but a concentrated band was always obvious about 3/4 of the way down the tube. If the sample contained a high proportion of antigen-stimulated cells, a second distinct band was observed in the top half of the tube. Fractions of 20 drops each were collected from the top of the gradient by enclosing the nitro-cellulose tube in a close fitting cylinder with a Teflon screw cap which forced a needle through the bottom of the tube. Bromobenzene, which is more dense than the albumin, was pumped in from the hole in the bottom and the gradient thereby displaced and collected from the top.

A standard curve was prepared relating density to the refractive index of the albumin (Figure II-1b). Density was determined by comparing the weight of glass weighing bottles filled with water or with various albumin solutions at room temperature. One drop of an albumin solution was sufficient to determine the refractive index using an Abbe refractometer. For each fraction from a gradient, it was then a simple matter to record the refractive index, as the presence of cells did not interfere with the reading. 0.1 ml from each fraction was used for determining the cell count and the percentage "large" cells. The remainder of the fraction was used for cell smears, plaque assays and electron microscopy.

Enzyme Assays

Acid Phosphatase · The method used was based on that developed by Bessey, Lowry and Brock (1946). In the reaction the substrate, p-nitrophenyl phosphate, is hydrolyzed by acid phosphatase to p-nitrophenol and inorganic phosphate. Addition of alkali stops the enzymic hydrolysis and forms a yellow p-nitrophenate derivative. The liberated chromagen is directly proportional to the acid phosphatase activity.

For the determination of 'free' enzyme in whole lymphocytes, 0.05 M citrate buffer pH 5.0 was used. The addition of sucrose was not necessary to protect the lysosomes from osmotic shock. 'Total' enzyme levels were determined in the presence of 0.1 per cent Triton X-100, the latter causing lysis of the cells and the lysosomal granules.

Lymphocytes (1-3 x 10^6 cells) were isolated by centrifugation and the lymph plasma was removed. 'Free' acid phosphatase was determined by adding 0.9 ml of citrate buffer and 0.1 ml of substrate. 'Total' acid phosphatase was determined by adding 0.9 ml of citrate buffer containing 0.1 per cent Triton X-100 and 0.1 ml of substrate. After a 15 minute incubation at room temperature, 4.0 ml of N/10 sodium hydroxide was added. Optical densities were read at 410 nm against a distilled water blank. A sample blank was prepared by adding the substrate after the alkali. This value, when detectable, was subtracted from the other readings.

Fresh samples of plasma, sera or lymph plasma were assayed by using 0.1 or 0.2 ml of sample, 0.8 or 0.7 ml of buffer and 0.1 ml of substrate. Incubations were done at 37°C for 30 minutes and then treated as for the lymphocyte samples.

β-glucuronidase · The assay was based on the method developed by Talalay, Fishman and Huggins (1946). The β-glucuronide of phenolphthalein was used as the substrate in 0.1 M acetate buffer, pH 4.6. β-glucuronidase, when present, liberates free phenolphthalein into the

medium. The hydrolysis is stopped and the colour developed by adding alkali to give a final pH of 10.5-10.7. For the determination of 'free' enzyme in whole lymphocytes, the buffer must be at pH 5.0 and contain 0.25 M sucrose to protect the lysosome granules from osmotic shock. 'Total' enzyme levels were determined in the presence of 0.1 per cent Triton X-100 in buffer at pH 5.0. 'Total' assays can be done at pH 4.6 when 'free' assays are not required.

Lymphocytes (about 1×10^7 cells) were centrifuged out of lymph and 0.9 ml of acetate buffer (containing Triton X-100 for 'total' assays) and 0.1 ml of substrate added. Incubations were done at $37^{\circ}C$ for 3 hours and the reaction was stopped by adding 2.5 ml of glycine buffer, pH 11.2. Precipitated protein was removed by centrifugation and the phenolphthalein colour read at 540 nm against a distilled water blank. Sample blanks were prepared by adding the substrate after the alkali. For plasma, sera or lymph plasma, 0.1 or 0.2 ml was made up to 0.9 ml with acetate buffer, pH 4.6 and 0.1 ml of substrate added. Incubations were done for 16 hours at $37^{\circ}C$ and then treated as for the lymphocyte samples. β-glucuronidase is very stable and assays were done on frozen plasma or cells that had been stored for a few days.

N-acetyl-β-D-glucosaminidase · The assay for this enzyme was based on methods described by Leaback and Walker (1961), Vaes and Jacques (1965) and Aronson and de Duve (1968). Acetyl glucosaminidase hydrolyses p-nitrophenyl glycoside in 0.1 M citrate buffer, pH 5.0. The reaction is stopped by the addition of glycine buffer, pH 10.7 and the colour of the aglycone developed.

For the determination of 'free' enzyme, 0.9 ml of citrate buffer containing 0.25 M sucrose and 0.1 ml of substrate were added to a pellet of about 5×10^6 lymphocytes. For 'total' assays the citrate buffer contained 0.1 per cent Triton X-100. Incubation was

carried out for 2 hours at 37°C and the reaction was stopped by the addition of 3.0 ml of glycine buffer, pH 10.7. Any precipitated protein was removed by centrifugation and the free p-nitrophenol was read at 410 nm against a distilled water blank. Sample blanks were prepared by adding the substrates after the alkali. For plasma, sera or lymph plasma, 0.1-0.2 ml of sample was combined with 0.8-0.7 ml of citrate buffer and 0.1 ml of substrate. These incubations were done at 37°C for 16 hours and then treated as for the lymphocytes.

Lactic dehydrogenase (LDH) . The method used was essentially that developed by Wroblewski and LaDue (1955). LDH catalyses the reaction

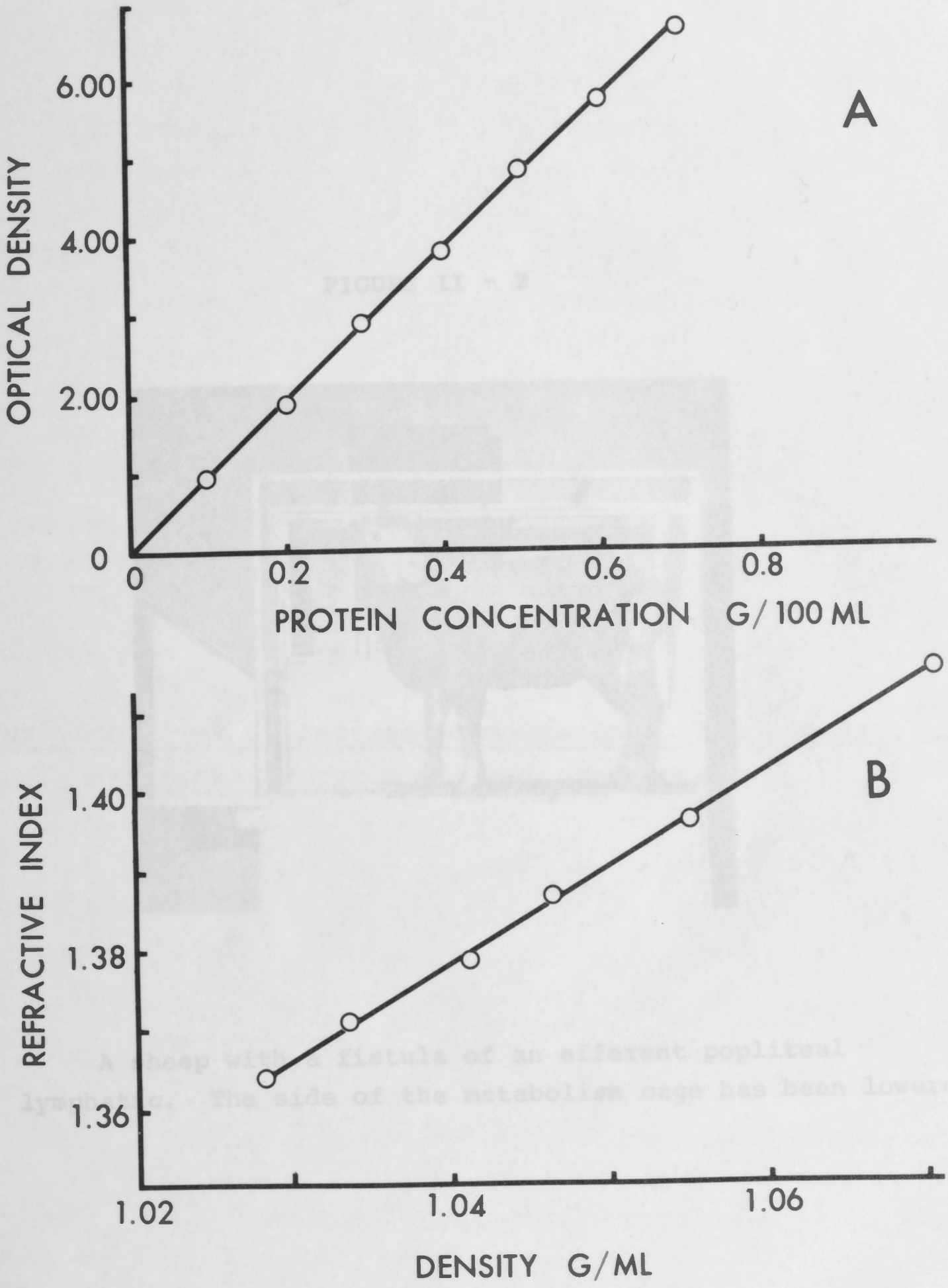
$$\text{L-Lactate} + NAD^+ \rightleftarrows \text{Pyruvate} + NADH + H^+,$$

where the equilibrium favours the formation of lactate and oxidised nicotinamide adenine dinucleotide (NAD^+). LDH activity is measured by the rate of reduction of pyruvate in the presence of reduced nicotinamide adenine dinucleotide (NADH). The rate of reaction is followed by the decrease in optical density at 340 nm due to the oxidation of NADH. A unit of activity is the amount of LDH which changes the optical density by 0.001 in 1 minute in a 3 ml assay mixture at 24-27°C. Lymphocyte LDH values were determined on extracts that were obtained either by sonicating the cells for 2 minutes at 4°C, or by subjecting cells to three cycles of freezing and thawing or by suspending the cells in 0.05 M phosphate buffer containing 0.1 per cent Triton X-100. Each method gave similar results in terms of enzyme activities.

0.02-0.10 ml of a lymphocyte extract, equivalent to 5×10^5-1×10^6 cells, was added to a cuvette followed by 2.93-2.85 ml of phosphate-pyruvate solution and 0.05 ml of NADH solution. Samples and solutions were incubated at 25°C before mixing. The optical density was read at 1 minute intervals for 3-5 minutes using a Pye Unicam SP8000 ultraviolet recording spectrophotometer. The reference cuvette contained phosphate-pyruvate solution. Plasma, serum and lymph plasma samples were done in

a similar manner. 2.85 ml of phosphate-pyruvate solution, 0.1 ml of sample and 0.05 ml of NADH solution were mixed thoroughly in the cuvette. If the change per minute was greater than 0.050 units the rate of change at 1 minute intervals was not linear and it was necessary to dilute the sample 1:5 or 1:10 with phosphate buffer.

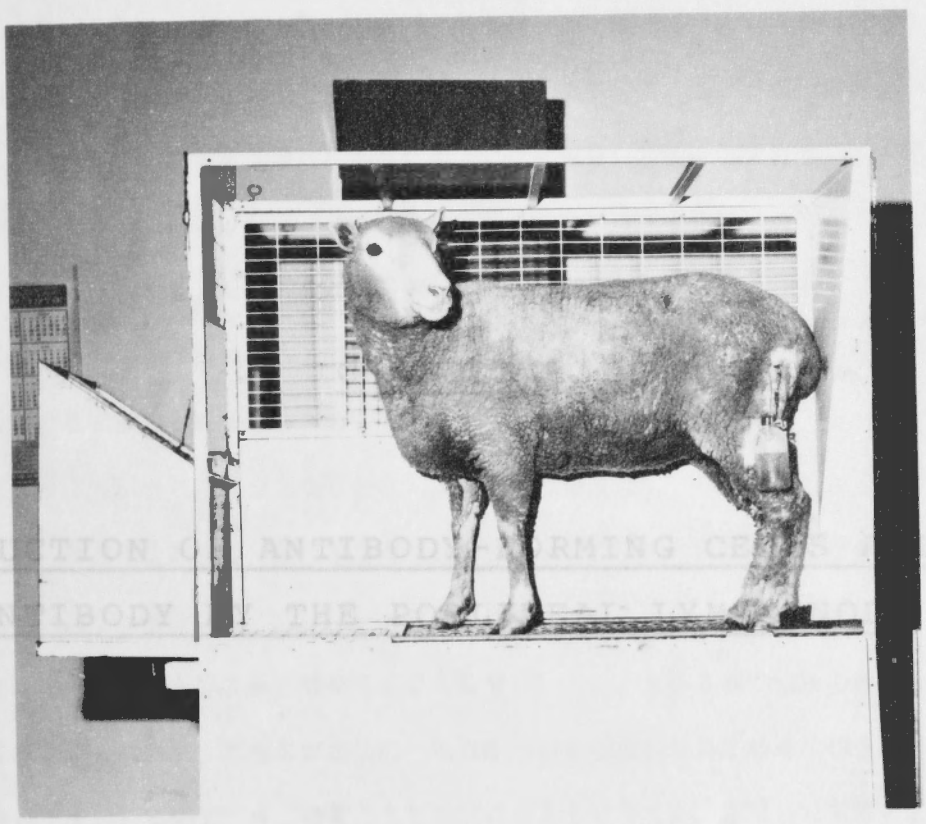
FIGURE II - 1



A. Standard curve relating protein concentration to optical density for Biuret assays.

B. Standard curve relating density of albumin solutions to refractive index.

FIGURE II - 2



A sheep with a fistula of an efferent popliteal lymphatic. The side of the metabolism cage has been lowered.

CHAPTER III

THE PRODUCTION OF ANTIBODY-FORMING CELLS AND HUMORAL ANTIBODY BY THE POPLITEAL LYMPH NODE

The Production of Antibody-Forming Cells and Humoral Antibody by the Popliteal Lymph Node

The immune response in sheep as it occurs in the efferent lymph from the popliteal lymph node has been described for several antigens (Hall and Morris, 1963; Cunningham, Smith and Mercer, 1966; Hall, Morris, Moreno and Bessis, 1967; Smith and Morris, 1970). For most antigens studied, the appearance of transformed cells in the lymph corresponded to the time when titratable antibody also appeared in the lymph and there was a good correlation between the amount of antibody extractable from the cells and the number of basophilic blast cells present. However, this was not always the case. After a primary challenge with swine influenza virus, Smith and Morris (1970) reported a substantial basophilic cellular response in the lymph which had disappeared several days before the antibody titre in the lymph plasma reached its peak. Almost all the antibody detectable against influenza virus was resistant to 2-mercapto-ethanol and was therefore, presumably IgG.

The experiments described in this chapter were designed to differentiate between the production of IgM and IgG antibodies in terms of the cellular events that occur in the efferent lymph from a single lymph node. An attempt was made to assay IgM antibody-forming cells (direct plaque assay) and IgG antibody-forming cells (indirect plaque assay) using the method outlined by Dresser and Wortis (1965) and Sterzl and Riha (1965). For the purpose of this work, the term IgM is used for the antibody that was found in the first protein peak recovered from a G-200 Sephadex column and the antibody that was reduced by treatment with 2-mercapto-ethanol. Antibody that was found in the second protein peak after Sephadex chromatography and that was resistant to 2-mercapto-ethanol treatment is referred to as IgG. No immunochemistry of the light chains of sheep immunoglobulins has been carried out.

Results

The Relationship Between the Antibody Released From Plaque-Forming Cells and the Antibody Titrated in the Lymph Plasma

The Release of Titratable Antibody From Plaque-Forming Cells in Vitro

EXPERIMENTAL

Although titratable antibody appeared in the lymph plasma at the same time as plaque-forming cells (PFC) to Salmonella appeared in efferent popliteal lymph (Cunningham, Smith and Mercer, 1966) a formal demonstration that the antibody titrated actually came from the plaque-forming cells of lymph was not shown. Therefore experiments were designed using large numbers of efferent lymph cells ($5 \times 10^8 - 2 \times 10^9$), obtained during both primary and secondary responses to Salmonella to show the relationship between plaque-forming cells and the release of antibody by cells *in vitro*. Sheep with popliteal lymphatic fistulae were challenged with Salmonella and the cells obtained from the lymph throughout the immune response. The cells were centrifuged out of the lymph plasma, washed twice with cold Eagle's medium, counted and re-suspended to a volume of 5 ml. in various media. The different media tested were Eagle's containing different concentrations of actinomycin D or puromycin, calf-serum, isologous lymph plasma and PBS. In all experiments control cultures in pure Eagle's medium were compared with the tested media. Other control cultures were maintained at $0^\circ - 4^\circ C$ instead of at $37^\circ C$. Incubation times extended up to 6 hours and samples were assayed for the amount of antibody released and for the number of plaque-forming cells at various time intervals up to 6 hours.

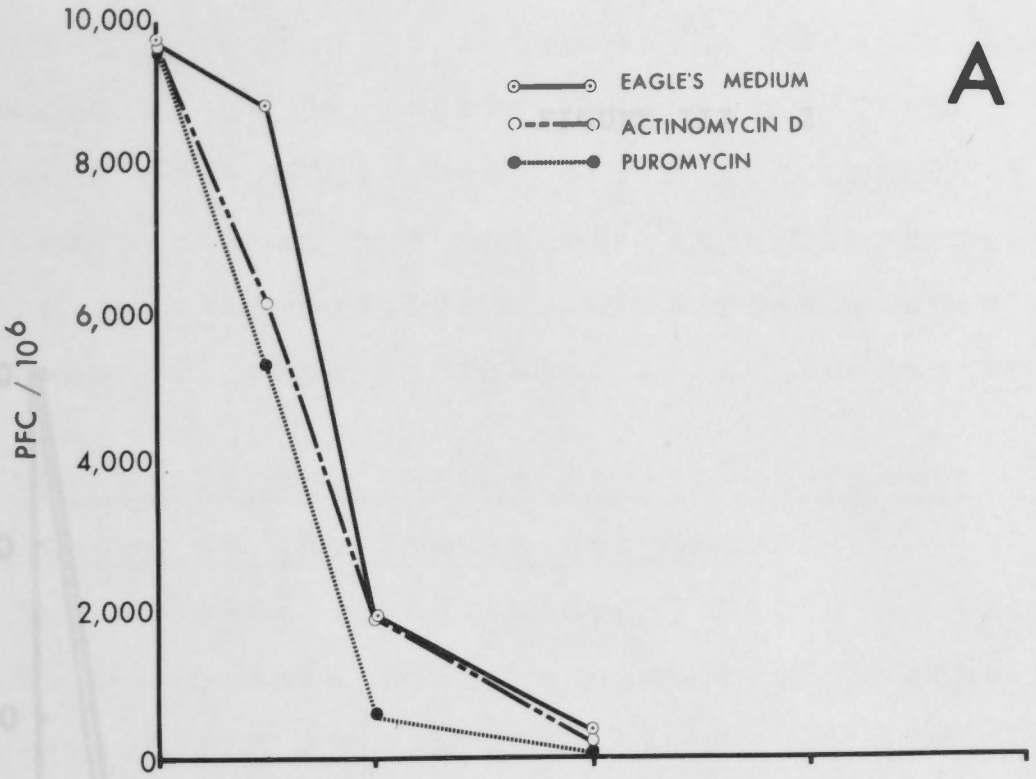
RESULTS

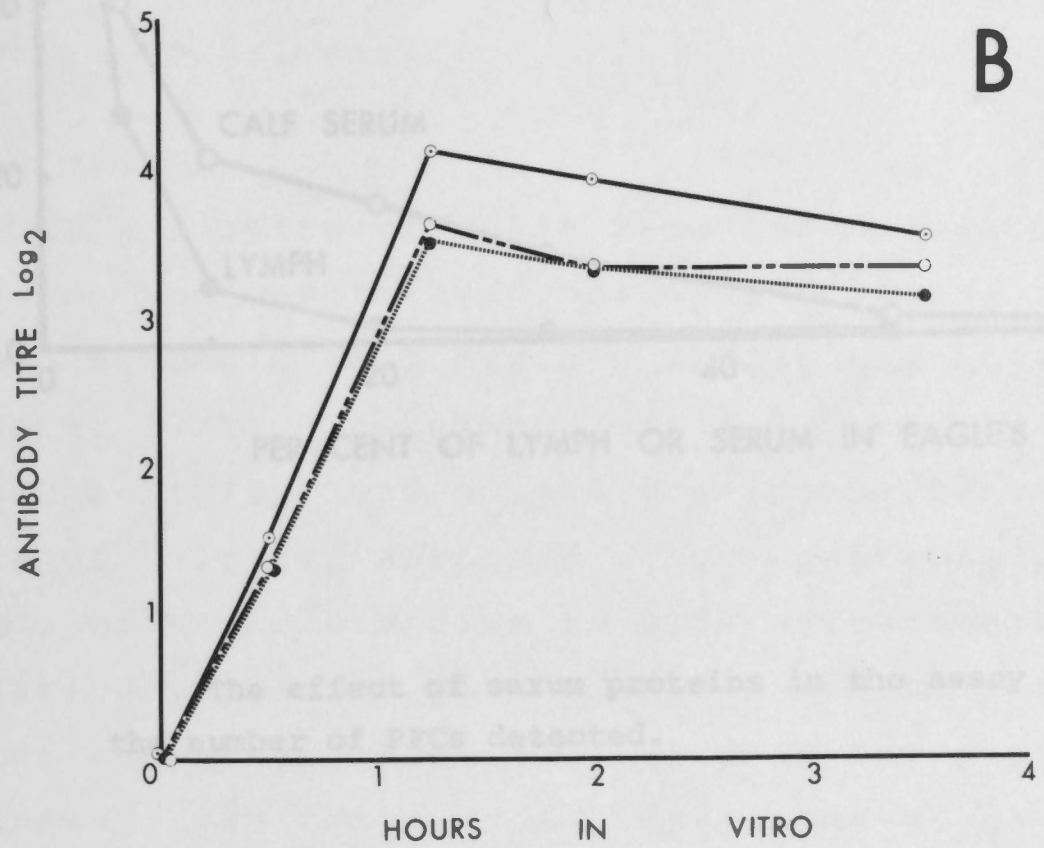
Antibody released from the cells was detected in the medium within 30 minutes. The highest titre that was obtained in this manner was 1:256. The maximum titre was reached after 2 hours of incubation and after this time, the titre either remained at a plateau or gradually declined. During the same period the number of PFC's in the cultures fell. Figure III-1 illustrates this relationship. This figure also shows the effect of actinomycin D (1 µg/ml) and puromycin (10 µg/ml) on the numbers of plaque-forming cells in culture and on the amount of antibody released. For the plaque-assays (Figure III-1a) each culture was done in triplicate and the standard deviations between the three groups of cultures overlapped. In the other experiment (Figure III-1b) where the antibody was titrated in the medium the cultures were set up in duplicate. The mean titres measured in those cultures to which various inhibitors were added were lower than in the control cultures but the difference was less than one serial dilution. In other experiments the effect of actinomycin D was tested at 0.1, 0.5, 5.0 and 10 µg/ml and puromycin was examined at 1 and 100 µg/ml. No significant suppression was found in either the antibody titre in the medium or in the number of PFC's detected when compared with cultures done in Eagle's medium alone. Although these assay methods would not detect small levels of protein synthesis, it was concluded that the PFC's released mainly preformed antibody when they were incubated under these conditions.

Other experiments showed that no titratable antibody was detected if the cells were incubated at 0° - $4^{\circ}C$. When either PBS or Hank's BSS were substituted for Eagle's medium, the titres of antibody in the medium were at least 2 tubes lower. The addition of serum proteins to the Eagle's medium also resulted in lower measurable titres. Figure III-2 shows that the addition of either isologous lymph or calf serum to the Eagle's medium reduced the number of PFC's detected.

The interpretation of these experiments was that the antibody titrated <u>in vitro</u> originated in the plaque-forming cells but that the antibody was preformed material that was

FIGURE III - 1

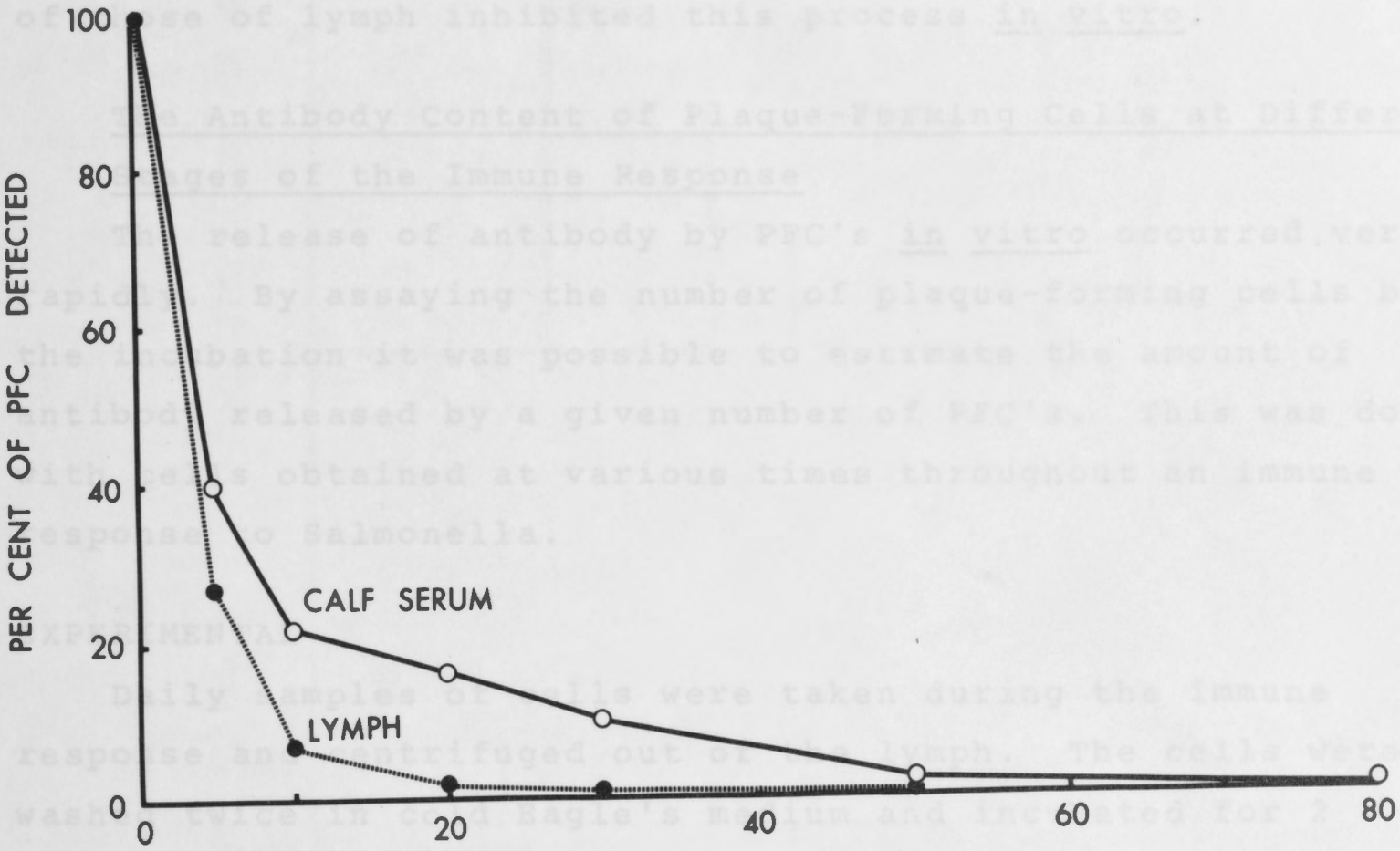




A. The number of detectable PFCs during a short term incubation at 37°C.

B. The titre of antibody in the culture medium during a short term incubation of PFCs at 37°C. Eagle's medium contained actinomycin D at 1 µg/ml or puromycin at 10 µg/ml. PFCs are represented as the mean of triplicate cultures and antibody titres are the mean of duplicate cultures.

FIGURE III - 2



The effect of serum proteins in the assay mixture on the number of PFCs detected.

released from the cells and probably not synthesized by them during the period of incubation. The release of the antibody appeared to be an active metabolic process since it did not occur at 0^{o} - 4^{o}C. Antibody was probably released in the circumstances of the culture experiment more rapidly than occurs *in vivo* because serum proteins even at levels of 20% of those of lymph inhibited this process *in vitro*.

The Antibody Content of Plaque-Forming Cells at Different Stages of the Immune Response

The release of antibody by PFC's *in vitro* occurred very rapidly. By assaying the number of plaque-forming cells before the incubation it was possible to estimate the amount of antibody released by a given number of PFC's. This was done with cells obtained at various times throughout an immune response to Salmonella.

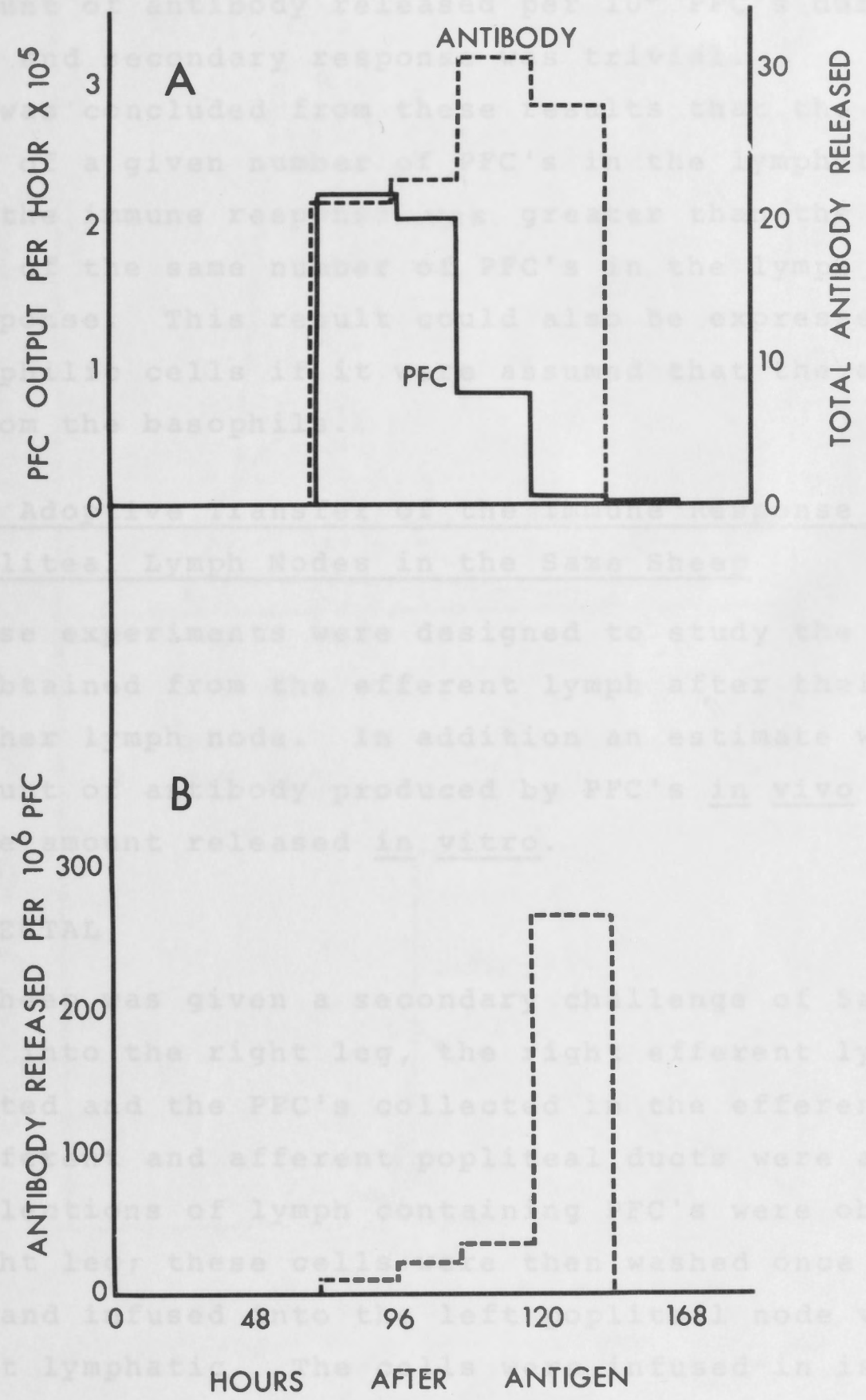
EXPERIMENTAL

Daily samples of cells were taken during the immune response and centrifuged out of the lymph. The cells were washed twice in cold Eagle's medium and incubated for 2 hours at 37^{o}C. The number of cells was counted and an assay of PFC activity done on the pre-incubated cell sample. The titre of released antibody ($\log_2$) was multiplied by the volume of Eagle's medium to give an estimate of the quantity of antibody present in the medium and this value was divided by the number of PFC's that were in the population of cells incubated. In this way a comparison was made between the antibody released by PFC's at different stages of the response.

RESULTS

Figure III-3 shows the output of PFC's from the popliteal node during a primary response in relation to the amount of antibody released by these cells *in vitro*. The total amount of antibody leaving the node contained within the lymph cells reached a maximum close to when the output of antibody-forming cells reached their peak (Figure III-3a). However, the amount of antibody released per 10^{6} PFC's increased as the response progressed (Figure III-3b).

FIGURE III - 3



A. The output of PFCs from the popliteal node during a primary immune response to Salmonella in relation to the total amount of antibody released in vitro by the PFCs.

B. The amount of antibody released per 10^6 PFCs.

Similar results were obtained during 2 other primary and 2 secondary responses to Salmonella. The differences between the amount of antibody released per 10^6 PFC's during the primary and secondary response was trivial.

It was concluded from these results that the antibody content of a given number of PFC's in the lymph towards the end of the immune response was greater than the antibody content of the same number of PFC's in the lymph early in the response. This result could also be expressed in terms of basophilic cells if it were assumed that the antibody came only from the basophils.

The Adoptive Transfer of the Immune Response Between Popliteal Lymph Nodes in the Same Sheep

These experiments were designed to study the fate of PFC's obtained from the efferent lymph after their transfer to another lymph node. In addition an estimate was made of the amount of antibody produced by PFC's *in vivo* compared with the amount released *in vitro*.

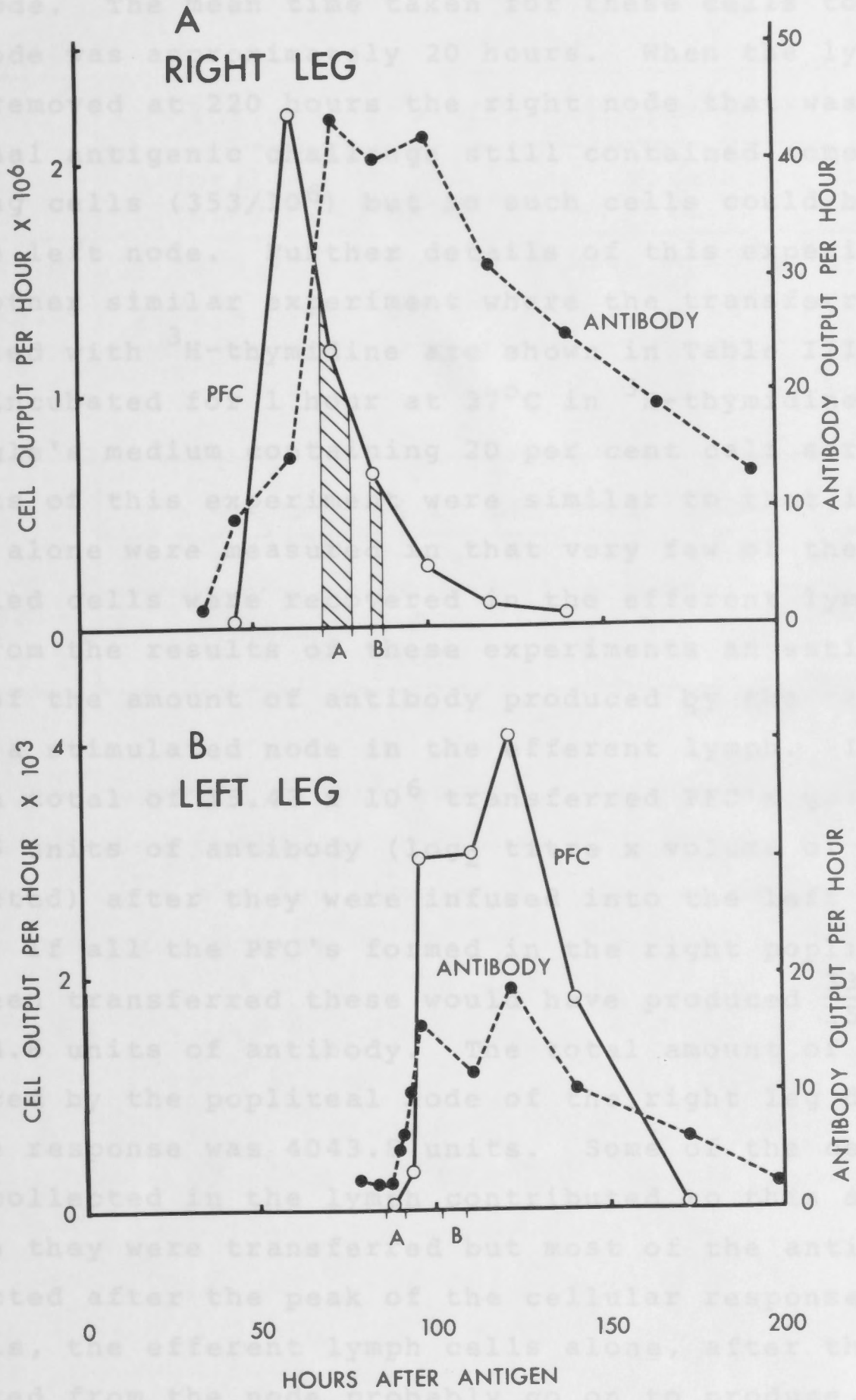
EXPERIMENTAL

A sheep was given a secondary challenge of Salmonella antigen into the right leg, the right efferent lymphatic cannulated and the PFC's collected in the efferent lymph. The left efferent and afferent popliteal ducts were also cannulated. Two collections of lymph containing PFC's were obtained from the right leg; these cells were then washed once in Eagle's medium and infused into the left popliteal node via the afferent lymphatic. The cells were infused in isologous lymph plasma that was free of antibody. Both PFC's and antibody were subsequently measured in the left efferent lymph.

RESULTS

Figure III-4 shows the results of this experiment. The left lymph node into which the PFC's were infused produced a substantial amount of antibody as shown by the antibody output in the efferent lymph. However, the number of PFC's which left the node and was recovered in the efferent lymph

FIGURE III - 4



A. An immune response to Salmonella. Cells collected during the times indicated by A and B were infused into the opposite politeal node.

B. The immune response produced by the transferred cells. Two infusions of cells were given at times A and B.

was only 1.1 per cent of the number that was infused into the node. The mean time taken for these cells to pass through the node was approximately 20 hours. When the lymph nodes were removed at 220 hours the right node that was given the original antigenic challenge still contained some plaque-forming cells ($353/10^6$) but no such cells could be detected in the left node. Further details of this experiment and of another similar experiment where the transferred cells were labelled with ^{3}H-thymidine are shown in Table III-1. Cells were incubated for 1 hour at 37°C in ^{3}H-thymidine (1μ Ci/ml) in Eagle's medium containing 20 per cent calf serum. The results of this experiment were similar to that in which PFC's alone were measured in that very few of the infused labelled cells were recovered in the efferent lymph.

From the results of these experiments an estimate was made of the amount of antibody produced by the cells that leave a stimulated node in the efferent lymph. It was found that a total of 15.43×10^6 transferred PFC's gave rise to 1152.5 units of antibody ($\log_2$ titre x volume of lymph collected) after they were infused into the left popliteal node. If all the PFC's formed in the right popliteal node had been transferred these would have produced $\frac{33.80}{15.43} \times 1152.5$ = 2524.6 units of antibody. The total amount of antibody produced by the popliteal node of the right leg during the entire response was 4043.8 units. Some of the cells that were collected in the lymph contributed to this antibody before they were transferred but most of the antibody was collected after the peak of the cellular response in the lymph. That is, the efferent lymph cells alone, after they have migrated from the node probably go on to produce, at a minimum, more than half the amount of antibody produced by the cells that remain behind in the node that received the challenge. It was estimated that the migratory cells which appeared in the lymph produced about 100 times more antibody *in vivo* over a period of 1 - 2 days than they would have released if they had been cultivated *in vitro*.

These calculations were based on the assumption that the antibody titrated in the left efferent lymph did come from the cells infused and not from the circulation, however blood

TABLE III-1

	Infused into Afferent Lymphatic			
	Total Cells	PFC /10^6	Total PFC	Labelled Cells
Exp.1	5.85×10^8	26,325	1.54×10^7	-
Exp.2	3.50×10^7	23,000	8.05×10^5	2.48×10^7

	Recovered in Efferent Lymph	
	PFC %	Labelled Cells %
Exp.1	1.10	-
Exp.2	1.05	2.60

The recovery of antigen-stimulated cells infused into an unstimulated popliteal lymph node. Cells were labelled with ^{3}H-thymidine and the number of positive cells was determined from autoradiographs.

antibody levels were not determined. It has been shown that when antigen is infused up an afferent lymphatic no systemic titre appears (Hall, Morris, Moreno and Bessis, 1967). When systemic antibody is present antibody titres in the lymph remain elevated for longer periods of time as the antibody passes from the blood steam into the lymph. Because the antibody curve in the left efferent lymph corresponded closely to the period of time when the PFC's were present in the lymph it was felt that the immune response in these experiments was confined to the two popliteal nodes.

The Indirect Plaque Assay

The Effects of Heterologous Antiserum on the Direct Plaque Assay

EXPERIMENTAL

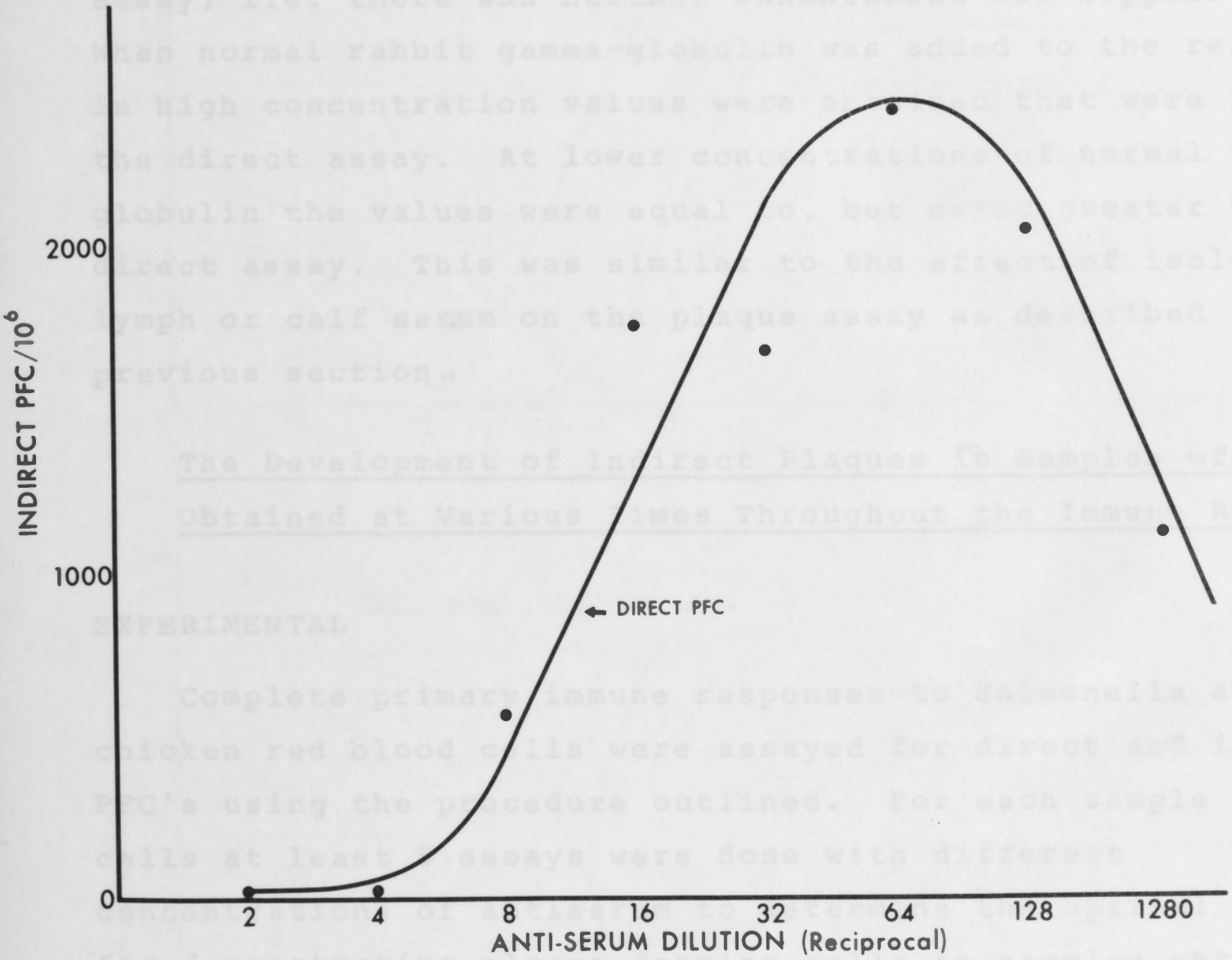
An antiserum which was capable of producing a visible precipitate when added to sheep lymph was raised in rabbits against sheep plasma proteins. The gamma-globulin fraction of this antiserum was obtained by fractionation with 18 per cent Na_2SO_4; this fraction was dialyzed against PBS and used in all the experiments.

For the direct PFC assays the reaction contained a mixture of sheep lymphoid cells, target erythrocytes and complement, in Eagle's medium. For the indirect PFC assays the stock gamma-globulin solution was diluted 1:64 with Eagle's medium and added to the reaction mixture in amounts varying from 0.1 per cent to 40 per cent of the final volume. Control assays were done with media containing gamma-globulins from normal, unimmunized rabbits.

RESULTS

Figure III-5 shows the number of PFC/10^6 cells detected in the presence of different concentrations of the antiserum (indirect plaque assay) in a sample of cells obtained from efferent popliteal lymph after challenge with Salmonella. In this experiment the direct assay carried out in the conventional way detected 962 PFC/10^6. The indirect assay

FIGURE III - 5



The number of indirect PFCs detected in the presence of rabbit anti-sheep serum.

on the other hand detected from 0 PFC/10^6 to 2,439 PFC/10^6 depending on the concentration of antiserum present in the reaction mixture. High concentrations of antiserum inhibited the assay and for this particular sample of cells a 1:64 dilution gave the maximum enhancement of the number of cells detected above the direct assay. Further dilutions of the antiserum resulted in values that were similar to the direct assay, i.e. there was neither enhancement nor suppression. When normal rabbit gamma-globulin was added to the reaction in high concentration values were obtained that were less than the direct assay. At lower concentrations of normal rabbit globulin the values were equal to, but never greater than the direct assay. This was similar to the effect of isologous lymph or calf serum on the plaque assay as described in the previous section.

The Development of Indirect Plaques in Samples of Cells Obtained at Various Times Throughout the Immune Response

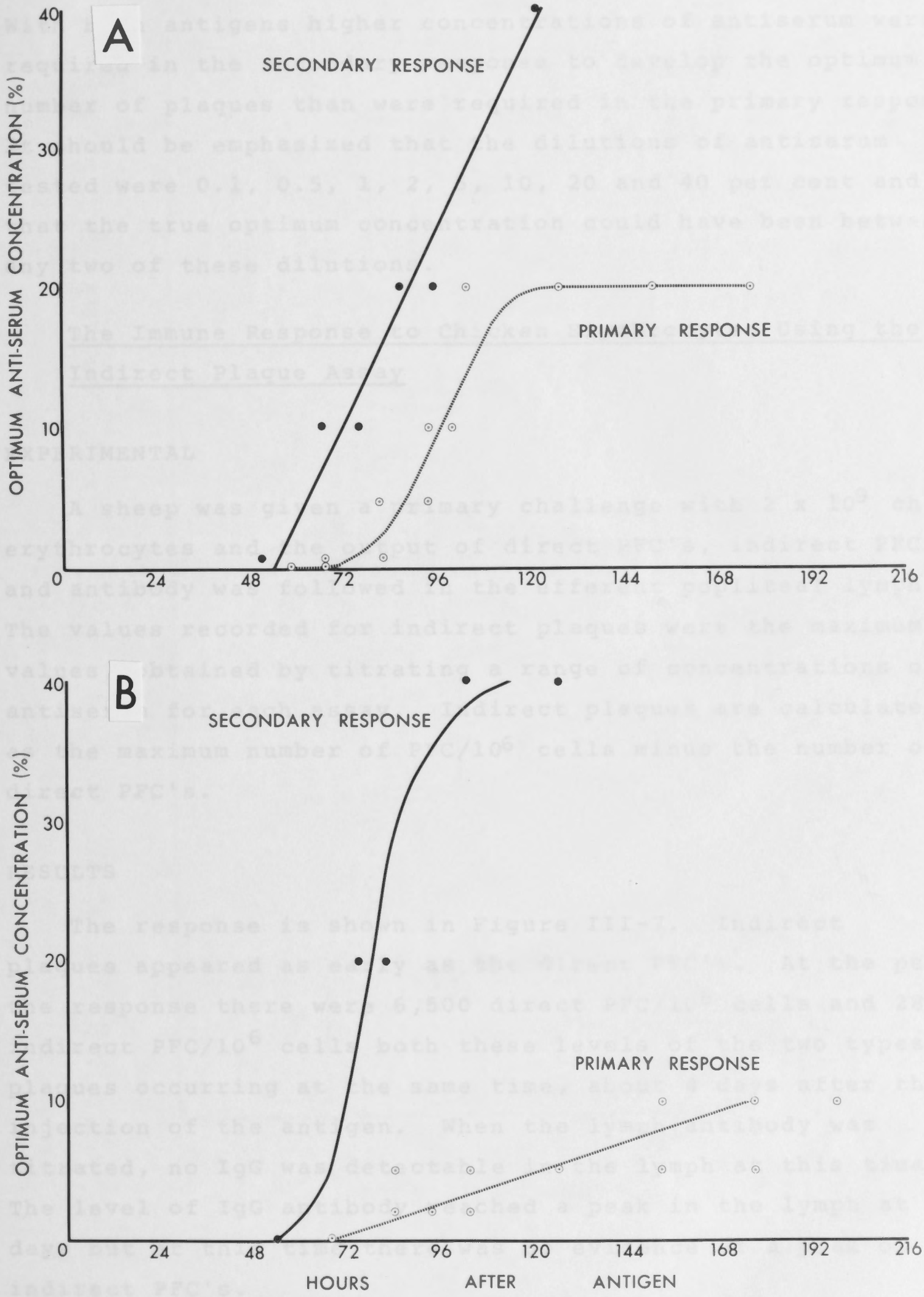
EXPERIMENTAL

Complete primary immune responses to Salmonella and chicken red blood cells were assayed for direct and indirect PFC's using the procedure outlined. For each sample of cells at least 5 assays were done with different concentrations of antiserum to determine the optimal conditions for demonstrating plaque-forming cells in samples obtained at various times after antigenic challenge.

RESULTS

The concentration of antiserum required to demonstrate the greatest numbers of PFC by the indirect assay changed as the immune response progressed. During the progression of a response the optimum concentration of antiserum required increased over a 100-400 fold range. Figure III-6a shows this effect during both primary and secondary responses to Salmonella. Each point on the graph represents a single assay done during 2 primary and 2 secondary responses. Figure III-6b shows that similar results were found for 3

FIGURE III - 6



The concentration of anti-serum required to develop the maximum number of indirect PFCs during immune responses to -

A. Salmonella

B. chicken erythrocytes.

primary and 1 secondary response to chicken erythrocytes. With both antigens higher concentrations of antiserum were required in the secondary response to develop the optimum number of plaques than were required in the primary response. It should be emphasized that the dilutions of antiserum tested were 0.1, 0.5, 1, 2, 5, 10, 20 and 40 per cent and that the true optimum concentration could have been between any two of these dilutions.

The Immune Response to Chicken Erythrocytes Using the Indirect Plaque Assay

EXPERIMENTAL

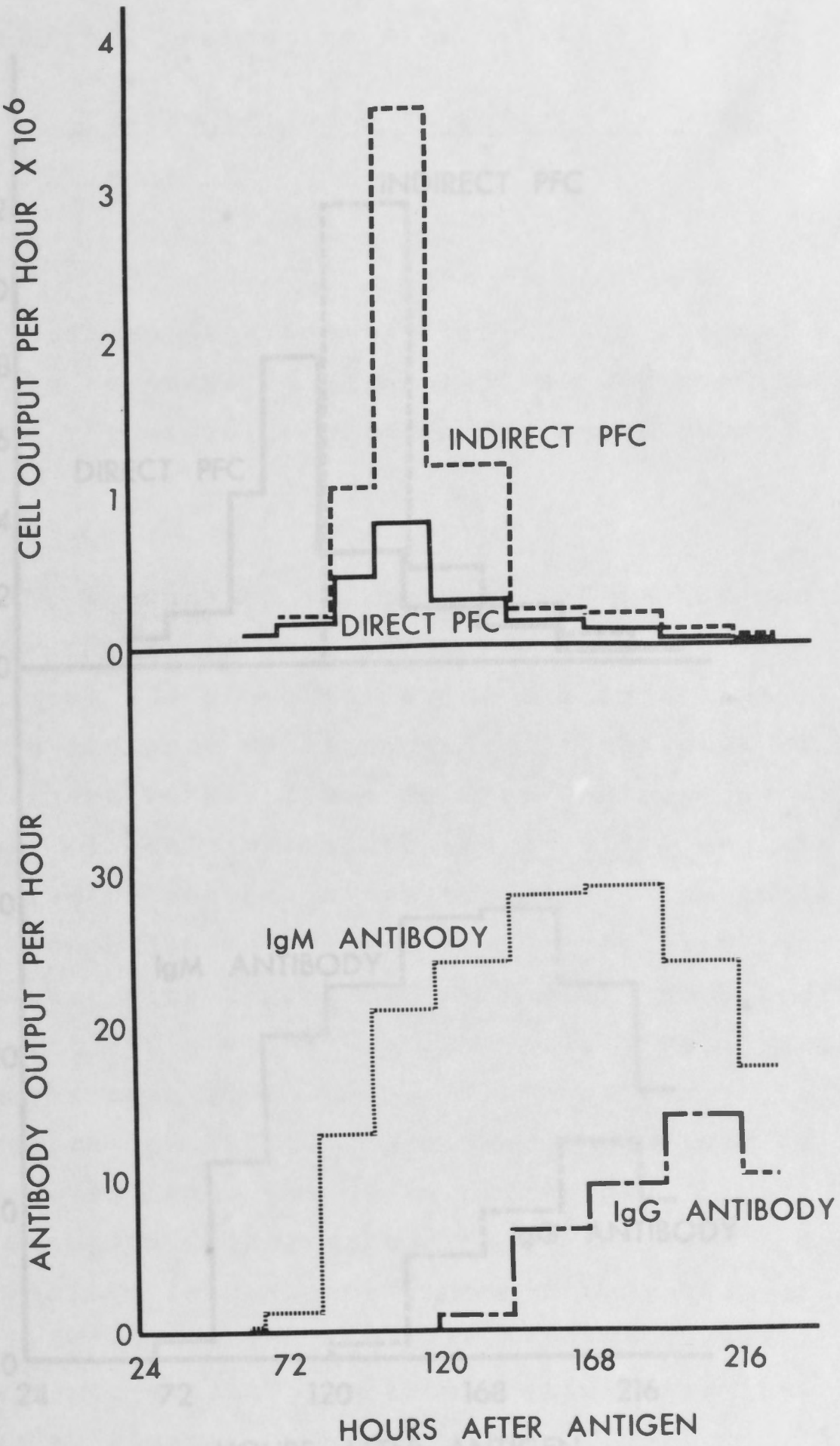
A sheep was given a primary challenge with 2×10^9 chicken erythrocytes and the output of direct PFC's, indirect PFC's and antibody was followed in the efferent popliteal lymph. The values recorded for indirect plaques were the maximum values, obtained by titrating a range of concentrations of antiserum for each assay. Indirect plaques are calculated as the maximum number of PFC/10^6 cells minus the number of direct PFC's.

RESULTS

The response is shown in Figure III-7. Indirect plaques appeared as early as the direct PFC's. At the peak of the response there were 6,500 direct PFC/10^6 cells and 28,430 indirect PFC/10^6 cells both these levels of the two types of plaques occurring at the same time, about 4 days after the injection of the antigen. When the lymph antibody was titrated, no IgG was detectable in the lymph at this time. The level of IgG antibody reached a peak in the lymph at 8 days but at this time there was no evidence of a peak of indirect PFC's.

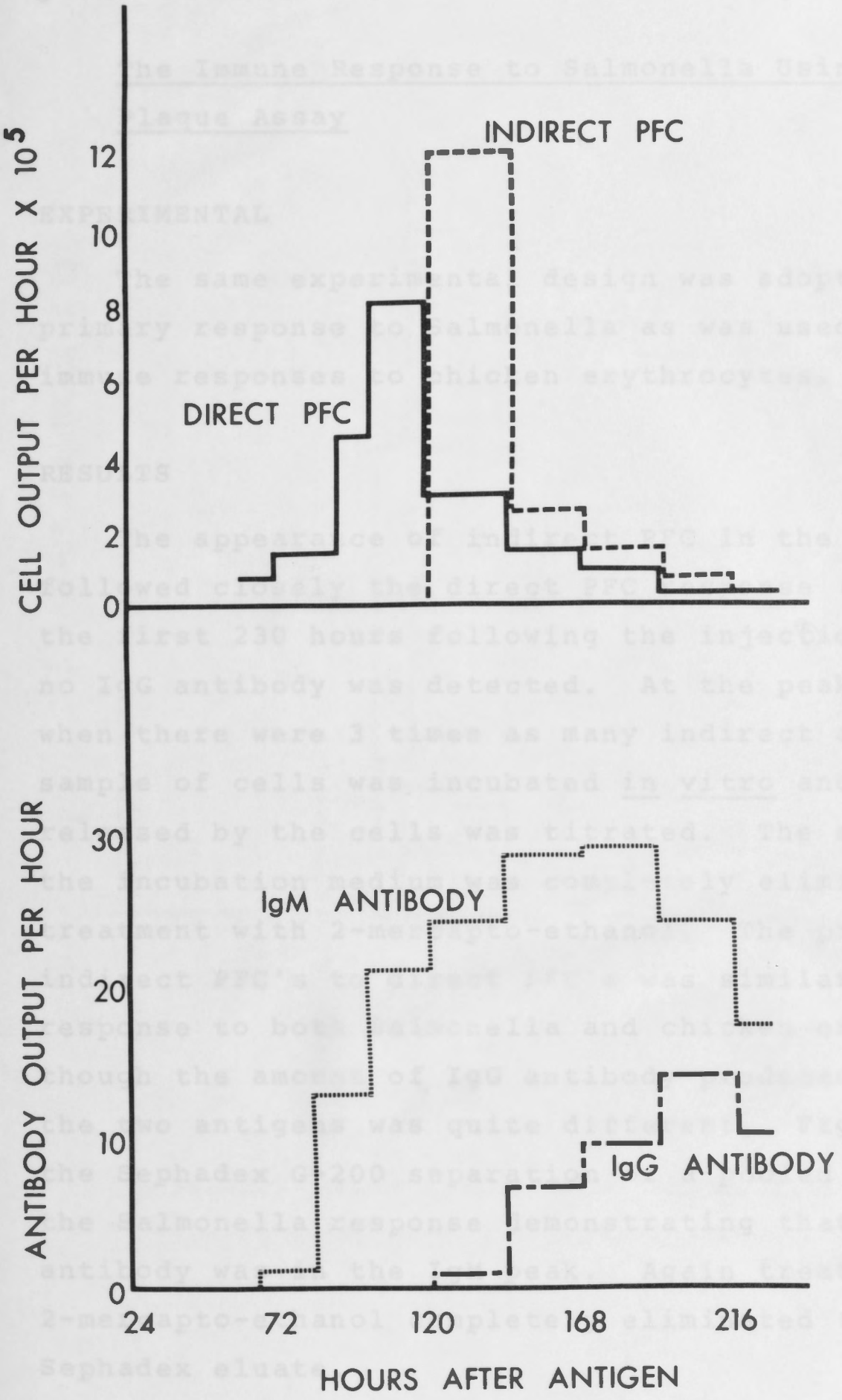
Figure III-8 shows the same response graphed in a different manner. If a single concentration of antiserum had been used (10 per cent) throughout the response, the majority of the indirect PFC's which appeared early in the response would not have been detected. Under these

FIGURE III - 7



A primary immune response to chicken erythrocytes showing the relationship between indirect PFCs and IgG antibody.

FIGURE III - 8



The same response to chicken erythrocytes as described in Figure III - 7 except that a single concentration of anti-serum was used throughout the response to assay indirect PFCs.

circumstances the response would have been misrepresented and it would have appeared that the indirect PFC's reached a peak at 130 hours, the time at which IgG antibody appeared.

The Immune Response to Salmonella Using the Indirect Plaque Assay

EXPERIMENTAL

The same experimental design was adopted to follow the primary response to Salmonella as was used to study the immune responses to chicken erythrocytes.

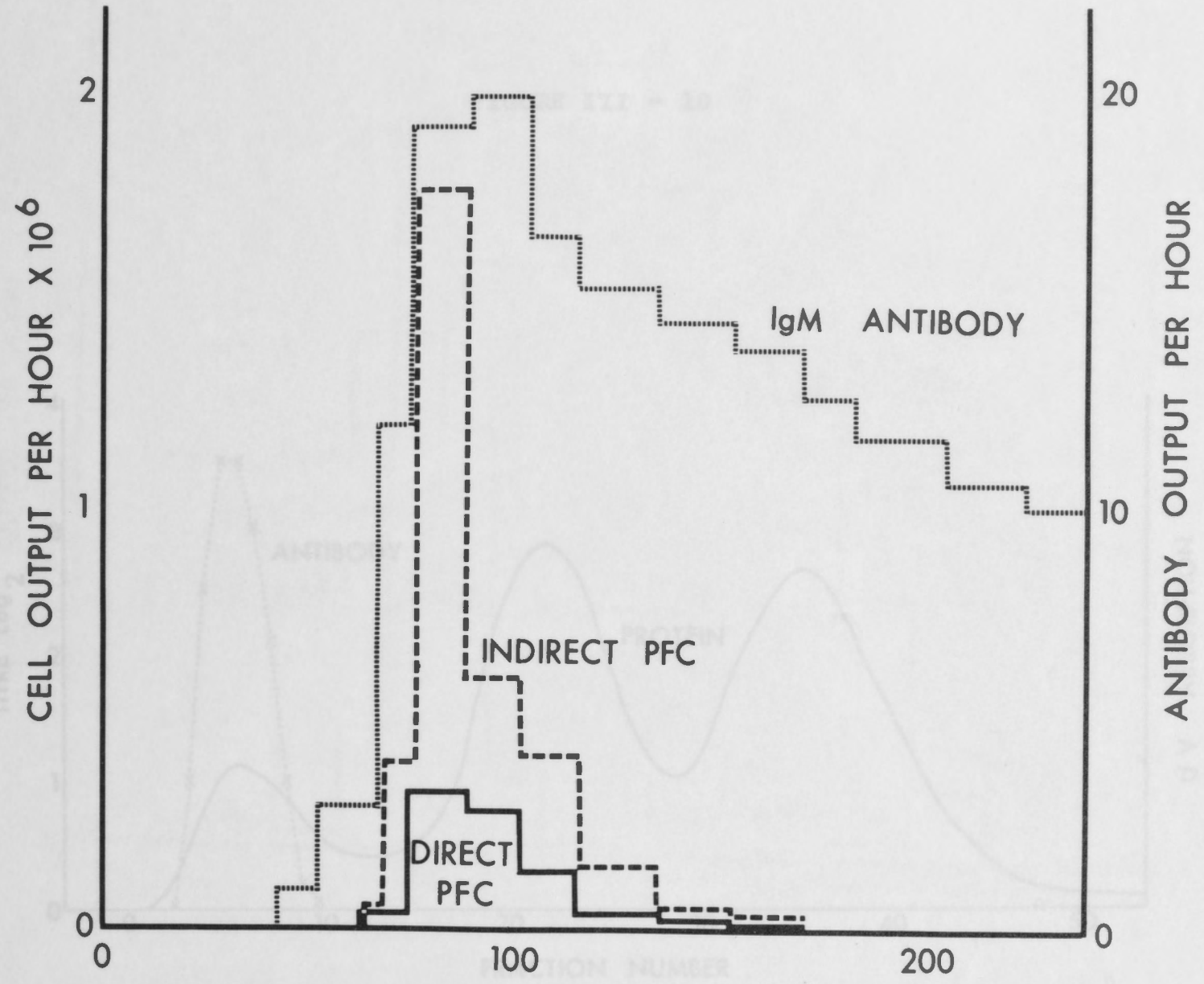
RESULTS

The appearance of indirect PFC in the popliteal lymph followed closely the direct PFC response (Figure III-9). Over the first 230 hours following the injection of the antigen no IgG antibody was detected. At the peak of the response when there were 3 times as many indirect as direct PFC's a sample of cells was incubated *in vitro* and the antibody released by the cells was titrated. The antibody present in the incubation medium was completely eliminated after treatment with 2-mercapto-ethanol. The proportion of indirect PFC's to direct PFC's was similar during the response to both Salmonella and chicken erythrocytes even though the amount of IgG antibody produced in response to the two antigens was quite different. Figure III-10 shows the Sephadex G-200 separation of a pooled lymph sample from the Salmonella response demonstrating that all the detectable antibody was in the IgM peak. Again treatment with 2-mercapto-ethanol completely eliminated this peak from the Sephadex eluate.

When IgG was detected during a Salmonella response it occurred very late, as a terminal event, long after the cellular response in the lymph had died away (Figure III-11).

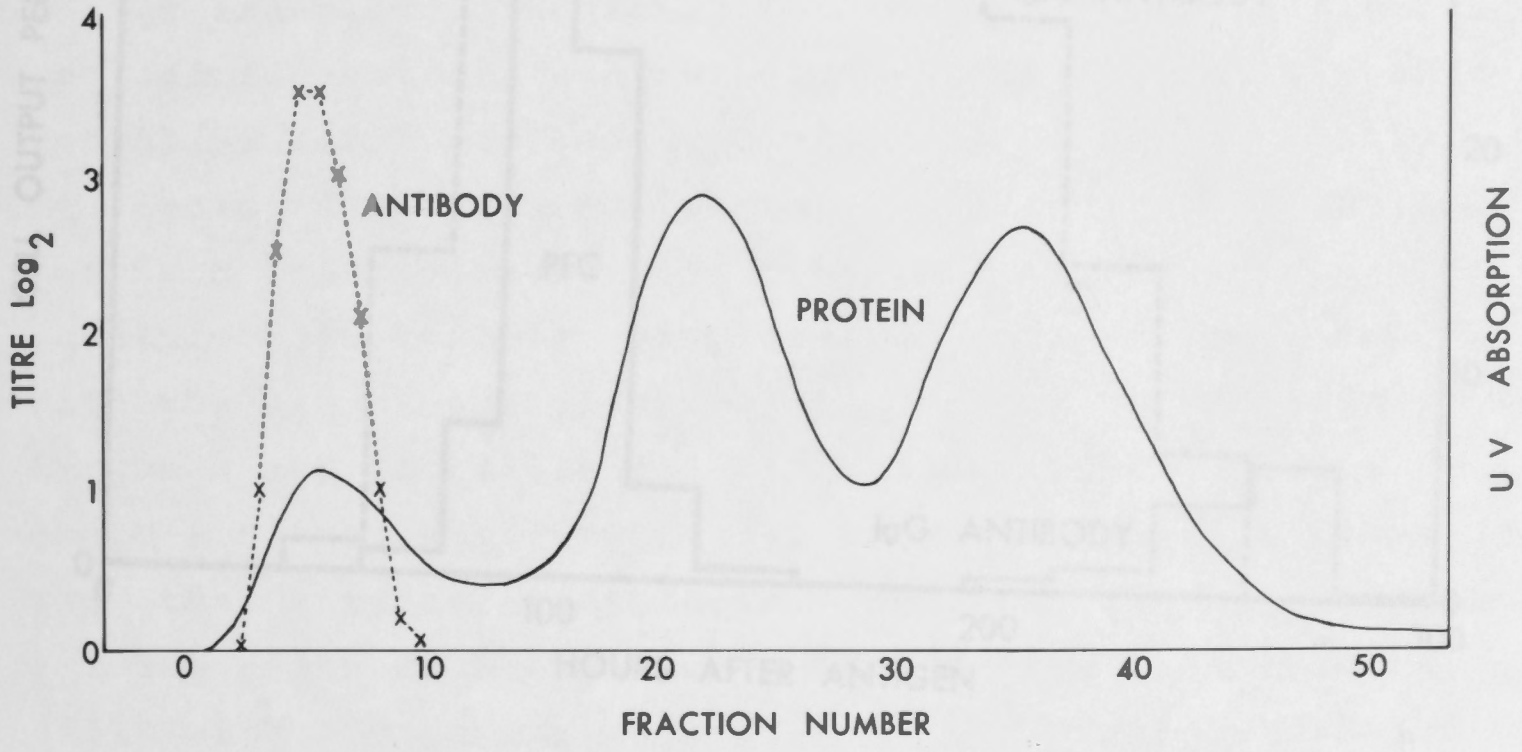
Thus the addition of heterologous antiserum enhanced the sensitivity of the direct PFC assay but it was concluded that the cells revealed as antibody-forming cells by this method were not those specifically forming IgG antibody.

FIGURE III - 9



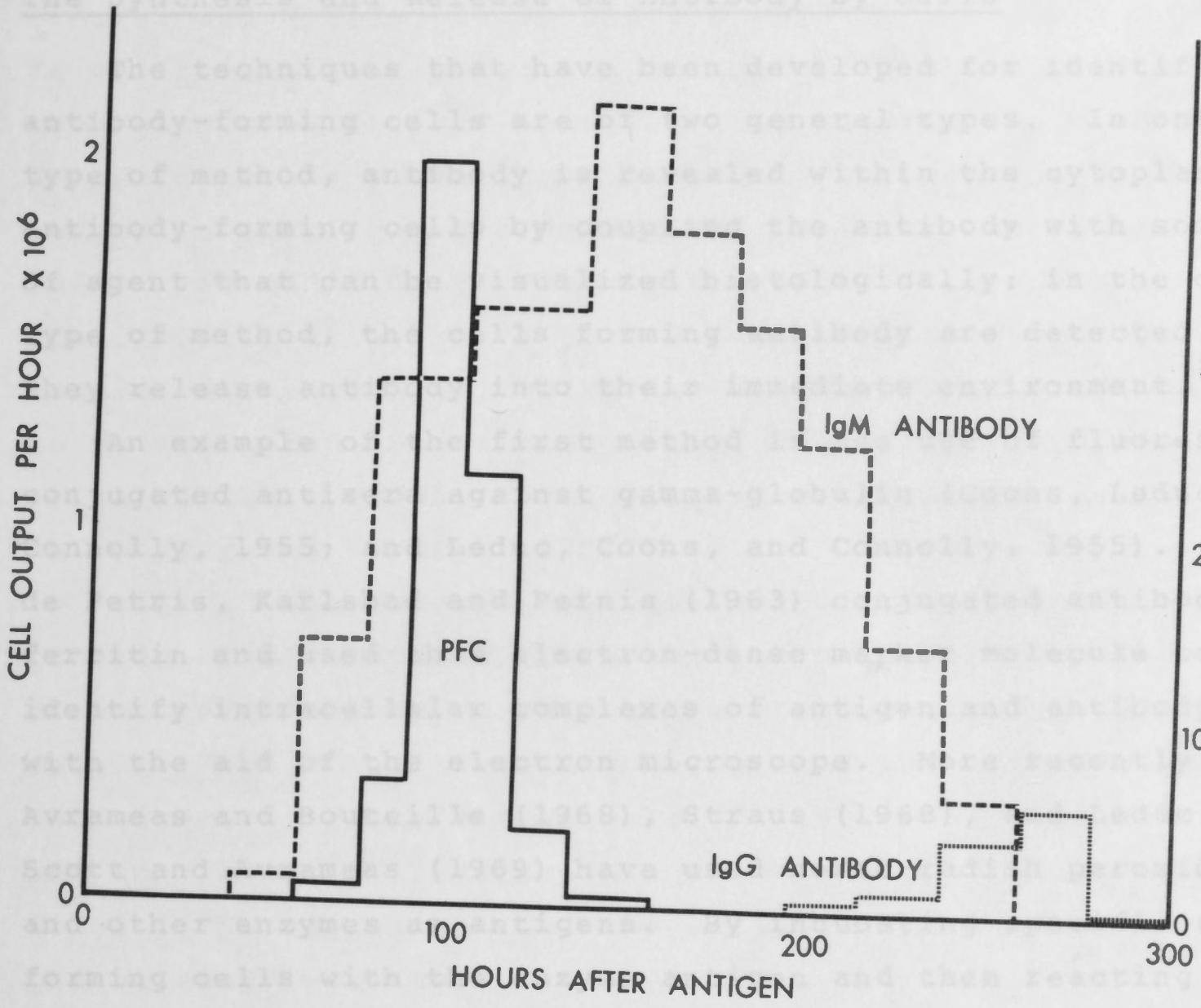
A primary immune response to Salmonella showing the relationships between direct PFCs, indirect PFCs and antibody.

FIGURE III - 10



The separation, using Sephadex G-200, of a pooled lymph sample obtained during a primary immune response to Salmonella.

FIGURE III - 11



A primary immune response to Salmonella showing the relationship between PFCs and IgG antibody.

Discussion

The Synthesis and Release of Antibody by Cells

The techniques that have been developed for identifying antibody-forming cells are of two general types. In one type of method, antibody is revealed within the cytoplasm of antibody-forming cells by coupling the antibody with some type of agent that can be visualized histologically; in the other type of method, the cells forming antibody are detected as they release antibody into their immediate environment.

An example of the first method is the use of fluorescein conjugated antisera against gamma-globulin (Coons, Leduc and Connolly, 1955; and Leduc, Coons, and Connolly, 1955). de Petris, Karlsbad and Pernis (1963) conjugated antibody with ferritin and used this electron-dense marker molecule to identify intracellular complexes of antigen and antibody with the aid of the electron microscope. More recently Leduc, Avrameas and Bouteille (1968), Straus (1968), and Leduc, Scott and Avrameas (1969) have used horse-radish peroxidase and other enzymes as antigens. By incubating specific antibody-forming cells with the enzyme antigen and then reacting the cells with the enzyme substrate in the presence of various substances, products are formed which can be identified by light and electron microscopy. Horse-radish peroxidase has also been conjugated with antibody and used to detect intracellular gamma-globulin by a method similar to the fluorescein or ferritin techniques (Avrameas and Bouteille, 1968).

The second method for identifying antibody-forming cells detects antibody that is released from the cell. Bacterial adherence to the surface of antibody-forming cells was described by Reiss, Mertens and Ehrich (1950), Hayes and Dougherty (1954) and Makela and Nossal (1961). Attardi, Cohn, Horibata and Lennox (1959) used an assay involving neutralization of bacteriophage by antibody secreted into the medium. The plaque assay for antibody-forming cells has been used extensively since its development by Jerne, Nordin

and Henry (1963) and Ingraham and Bussard (1964). Modifications of the original assay methods have increased its sensitivity (Cunningham, 1965; Sterzl and Riha, 1965; Dresser and Wortis, 1965) and the ease with which it can be performed (Cunningham and Szenberg, 1968), and techniques have also been devised to enable the assay to be used for detecting cells producing antibody to antigens other than red cells (Halliday and Webb, 1965; Walters and Jackson, 1968). In addition to these extensions of the original assay, other modifications have been developed which apparently enable cells producing different classes of immunoglobulins to be evaluated (Sterzl and Riha, 1965; Pernis, Chiappino, Kelus and Gell, 1965; Wortis, Dresser and Anderson, 1969; Sell, Park and Nordin, 1970).

Most of these techniques are useful as assays only and provide little information about the mechanisms of secretion of antibody as they operate under physiological conditions. Jerne et al (1963) were unable to show any effect of various inhibitors of protein synthesis on the development of plaques but contradictory results were described by Ingraham et al (1964), who suggested that the antibody measured by the plaque assay was newly synthesized.

The results described in this chapter showed that when a population of cells containing PFC's was incubated in a culture medium at 37°C, the number of detectable plaques fell off rapidly regardless of whether actinomycin D or puromycin were present in the medium. In addition, the level of titratable antibody increased in the medium at a rate that was inversely proportional to the fall in the PFC activity. It was concluded that under these conditions, antibody released by the cells was being assayed and that this antibody was not synthesized de novo by the cells while they were in the culture medium. This result does not necessarily mean that no antibody synthesis was occurring in these cells, in fact, there is ample evidence that de novo antibody synthesis can occur in culture (Dutton, 1967). Rather it would seem that the amounts of newly synthesized antibody were probably smaller than could be detected by the methods employed.

Cells suspended in protein-free medium or medium with low concentrations of protein released their antibody more easily than did cells in a medium containing a high concentration of protein. The reason for this may lie in the fact that the cell membrane becomes more permeable in a protein-free medium or that the cells were damaged under these conditions. No experiments were done to test if the cells which had released their antibody in culture could subsequently recover and continue to divide and synthesize antibody upon transfer to another node *in vivo*.

Whether the concentration of protein that occurs in the blood and lymph regulates the release of antibody in a way similar to that observed under the present *in vitro* conditions is not known. When antibody-forming cells from lymph were transferred to another node, antibody production by the cells or their progeny continued for 1-2 days and they formed about 100 times more antibody than when they were incubated *in vitro*. There are several possible explanations for this finding.

Some of the cells were detected in the efferent lymph from the node into which they had been infused after 1-2 days, so they continued to synthesize and release antibody for a longer period of time than they did *in vitro*. Only 1 per cent of the infused cells were recovered in the efferent lymph and there was no evidence that any further amplification of the immune response was extended by these cells beyond this lymph node. Hall, Morris, Moreno and Bessis (1967) challenged the popliteal node of sheep with human red cells and cannulated the lumbar lymphatic vessel. The immune response in the lumbar lymph was much greater in terms of the blast cell output than could be accounted for by the response of the popliteal node alone, and it was concluded that the lumbar nodes were actively involved in the response. When the cells from the efferent popliteal lymph were killed and subsequently returned to the animal together with the lymph the systemic antibody titre only increased by 1 tube. The transport of antigen to the lumbar nodes via the efferent popliteal lymph would probably have contributed

to the stimulation of the lumbar nodes. The antigen could have been in the form of haemoglobin from the red cells either within cells or free in the lymph even though it was felt by these authors that the red cell stroma was removed by the popliteal node. In the present experiments, it seems unlikely that any significant amount of antigen was transferred. If it had been, the infused node would have responded at 3-4 days. Instead, the antibody-forming cells appeared in the efferent lymph within 10 hours of the infusion and the response was virtually over by 2 days. It is possible that antigen continues to regulate the synthesis of antibody in a cell even after the cell has started to secrete antibody. For this reason, the transfer of cells without antigen might be a somewhat artificial situation.

Quantitation of Immune Responses

The magnitude of an immune response is usually measured by the titre of specific antibody that occurs in the blood stream following the injection of antigen and is usually detected with assay systems in which antibody combines with antigen. However, some types of antibody have a higher avidity for antigen when compared with others (Eisen and Siskind, 1964) and this may be related to the class of immunoglobulin produced in the response. The ability of antibody to fix complement may also be crucial in determining how effective the antibody is in the body or in the assay system. Where titres are derived by serial dilutions of antibody-containing media, problems often arise when there is excess of either antigen or antibody and spurious results can be obtained.

The number of detectable antibody-forming cells is also used widely in estimating the magnitude of an immune response; however, this assay can also give misleading results unless the limitations of the technique are appreciated. It is usually not possible to measure the antibody content of a single cell nor is it possible to decide whether any cell has produced a quantity of antibody before it was examined

or whether it would have gone on to make a quantity of antibody had it not been subjected to the assay. The immune response as measured in the efferent lymph from a lymph node has some advantages in this regard. The titre of the antibody can take into account the lymph flow rate as this can be collected quantitatively; and as all the antibody produced in the node passes into the lymph, it is possible to estimate the amount of antibody released per hour from an individual node. The total number of antibody-forming cells that leave a node throughout the entire immune response can also be measured. The magnitude of an immune response can be estimated by counting the number of transformed cells without recourse to antigen dependent assays. Such an estimate is useful when the injected material is a mixture of antigens.

Examination of the antibody content of PFC's at different stages of the immune response revealed that there was variation between populations of cells coming from the same lymph node at different times. Furthermore, the populations of PFC's towards the end of a response contained approximately 20 times more antibody per cell than did PFC's appearing earlier in the response. Estimates of the number of PFC's/10^6 total cells or PFC's/spleen would not discriminate between these differences in antibody content. Where possible antibody titres as well as antibody-forming cells should be assayed together when attempting to quantitate immune responses.

Indirect Plaque-forming Cells and the Production of IgG Antibody

The enhancement of the PFC assay by heterologous antiserum was first reported by Dresser and Wortis (1965) and Sterzl and Riha (1965). The plaques that are developed in these circumstances have been referred to variously as facilitated, developed, indirect plaques or 7s or IgG plaques. Many workers (eg. Sterzl *et al* 1965; Wortis, Taylor and Dresser, 1966; Eidinger and Pross, 1967; Moller, 1968; Holtermann and Nordin, 1968; Chaperon, Selner and Claman, 1968; Uyeki

and Klassen, 1968; Malakian and Schwab, 1968; Nordin, 1968; Ceglowski and Friedman, 1968; Larson, Baker and Smith, 1968; Cunningham, 1968; Sell, Park and Nordin, 1970) have interpreted these plaques as evidence of IgG antibody-forming cells. In most of these studies, heterologous antibody to whole serum or the gamma-globulin fraction has been used to develop the plaques. Some of these studies, particularly with mice, have employed anti-γ-chain sera specific for IgG while Sell et al (1970) have used specific antisera to detect five classes of mouse immunoglobulin producing cells. Development of indirect plaques with class specific antisera is the best evidence that the cells are in fact producing different classes of immunoglobulins, although this is by no means proven. More crude preparations of antiserum must also contain IgG specific antibody which should have the same effect as the purified antisera. In most studies in which class specific antisera have been used, the antibody-forming cells have not been correlated with the appearance of class specific antibody.

Another argument used to support the concept that the indirect plaque assay is in fact demonstrating IgG antibody formation is that IgG antibodies have a low haemolytic capacity compared with IgM antibody (Humphrey, 1965). It has been reported that direct plaques are due to IgM antibody which is efficient at inducing haemolysis and that only one molecule of such antibody may be required to lyse a given red cell in the presence of complement (Borsos and Rapp, 1965). On the other hand, many more molecules of IgG antibody are said to be required to fix complement and lyse a cell (Humphrey, 1967). The action of the antiserum is apparently to promote haemolysis that otherwise would not occur. However, using mice, Poltz, Talal and Asofsky (1968) concluded that the concept of the indirect PFC representing an IgG producing cell was no longer tenable because they found indirect plaques produced by cells releasing IgM of low haemolytic efficiency.

A further argument relating indirect plaques to IgG production has also been contradicted. The immune response to most antigens involves the rapid appearance of IgM antibodies followed by the appearance of IgG antibodies

directed against the same antigen (Svehag and Mandel, 1964; Uhr Finkelstein and Baumann, 1962; and Sell, 1965). Studies in which it has been shown that the number of direct plaques first reaches a peak and is then followed by a peak of indirect plaques gives a circumstantial association of indirect plaques with IgG production (Sterzl *et al* 1965; Dresser *et al* 1965). However, it should be stressed that this evidence is only circumstantial. Uyeki *et al* (1968) have used a microlitre titration method to compare the haemolysins produced by PFC's with the haemolysins present in the serum. They showed that the peak of "developed" PFC's appeared 1 week prior to the peak serum levels of "developed" haemolysin. The reason for this discrepancy was not determined but the most obvious interpretation would be that the two events are unrelated.

The experiments described in this chapter showed that there was no correlation between the appearance of indirect plaques and IgG in the lymph for either of the two antigens studied. During a response to Salmonella, no IgG was detected but the enhancement of the plaque assay was the same order or magnitude as was seen during the response to chicken erythrocytes where a considerable amount of IgG was detected. Even when the indirect PFC's outnumbered the direct PFC's by 3 to 1, only IgM could be detected in the antibody that was released by the cells both *in vitro* and *in vivo*. The number of indirect plaques detected could be enhanced or depressed by manipulating the concentration of the antiserum used in the assay system. This effect has also been reported by others (Dresser *et al* 1965, Sterzl *et al* 1965, L'age-stehr and Herzenberg 1970 and Sell *et al* 1970) when either class specific or mixed antisera were used. In view of the fact that the PFC's can be suppressed by either isologous or heterologous serum, there was no need to postulate that this suppression was a specific effect of antiserum. A feature that has probably been overlooked by others is that the "equivalence zone" of antiserum concentration required to develop the maximum number of plaques increases as the response progresses. This phenomenon

was described during the PFC response in rabbits by Chou, Cinader and Dubiski (1967). In the experiments of others the "optimum" concentration of antiserum has usually been determined only once, using cells obtained at a single time interval of the immune response. By using concentrations of antiserum that are too high, the detection of early indirect PFC's cannot be observed and the apparent peak of the indirect PFC response will be later than the peak of the direct PFC response. When the optimum concentration was determined for each assay, the direct and indirect PFC responses coincided.

The mechanism of enhancement of the direct plaque assay by heterologous antiserum may be related to the amount of antibody within the cell or on the surface of a cell, although not necessarily the class of the antibody. Chou *et al* (1967) suggested that the reason the optimum concentration of antiserum increased as the response progressed may have been related to an increasing amount of antibody within the antibody-forming cells. This suggestion is in line with the findings in the present experiments where both the intracellular antibody and the optimum antiserum concentrations increased as the response progressed. Another explanation for the enhancement of the assay has to be considered. Antibody-forming cells probably have antibody on their surface and if a heterologous antibody is then attached and complement fixation occurs, it is possible that lysis of the antibody-forming cell itself could occur with the subsequent release of intracellular antibody. This antibody may then be sufficient to form a plaque which otherwise would not have been detected. Examination of indirect plaques with high magnification microscopy did reveal that many of the lymphoid cells in the centre of the plaque were in fact in a damaged state.

The cellular basis of IgG production still remains unclear. There was no proliferative cellular response in the efferent lymph at the peak of an IgG response to Salmonella or chicken erythrocytes. The production of IgG appeared to be a terminal event in that the IgM antibody dropped rapidly as

the IgG first appeared. The feedback inhibition of IgG on IgM synthesis has been described and reviewed by Uhr and Möller (1967); however other mechanisms involving cell differentiation may just as readily explain this phenomenon. Schoenberg, Rupp and Moore (1964) have suggested that IgM is produced primarily by blast cells while plasma cells produce IgG, and this explanation is in line with the events in the primary immune response to influenza virus (Smith and Morris, 1970). This however, is probably not the whole story, as the secondary response to horse-radish peroxidase appears to be almost entirely an IgG response and essentially none of the antibody-forming cells in the lymph are plasma cells (See Chapter V).

Indirect plaques have not been assayed in the experiments described in subsequent chapters because of the tedious nature of the assay and because of the finding that this assay does not really differentiate the synthesis of different classes of antibody. Although it increases the sensitivity of the direct assay, several determinations using different concentrations of antiserum must be done on each cell sample and even then the optimum concentration may fall between two serial dilutions. Therefore, the errors tend to be greater than for the direct assay.

Summary

1. An estimate of the antibody content of cells found in lymph after antigenic challenge was made by titrating the amount of antibody that the cells released *in vitro*. It was concluded that the antibody so titrated came from the plaque-forming cells and that individual plaque-forming cells appearing in lymph towards the end of the immune response contained more antibody than the plaque-forming cells appearing earlier in the response.

2. It was estimated that the antibody-forming cells and their progeny produced about 100 times more antibody in the animal than they released when incubated *in vitro*.

3. With the aid of an antiserum, the sensitivity of the direct plaque assay could be increased. The optimum concentration of antiserum required to develop these indirect plaques increased as the response progressed.

4. It was concluded that the indirect plaque-forming cells were not producing IgG.

CHAPTER IV

THE SEPARATION OF ANTIGEN-STIMULATED CELLS USING ALBUMIN EQUILIBRIUM-DENSITY GRADIENTS

The Separation of Antigen-Stimulated Cells Using Albumin Equilibrium-Density Gradients

Hall, Morris, Moreno and Bessis (1967) and Smith and Morris (1970) suggested that cells produced in the antigenically stimulated lymph node comprise a residential population of cells, whose function appears to be the production of antibody locally in the lymph node and a population of antibody-forming cells that leave the lymph node by way of the efferent lymph duct. Some of these free floating cells can secrete antibody (Cunningham, Smith and Mercer, 1966), and there is evidence to suggest that they are responsible for the extension of the immune response to other lymph nodes and also for the spread of systemic immunity (Hall *et al* 1967) and immunological memory (Smith, Cunningham, Lafferty and Morris, 1970). There thus appears in efferent lymph following an antigenic challenge cells with a variety of specific immunological functions in addition to a background of cells that do not appear to be concerned with the particular immune response.

The experiments in this chapter are concerned with technique. They were designed to separate the population of cells in the lymph producing antibody from the other cells found in lymph and in lymph nodes and to compare their cytology. The technique employed for this fractionation was similar to that described by Shortman (1968).

Approximately 15 gradients were examined using efferent lymph cells from both normal nodes and stimulated nodes taken from five different sheep. Familiarity with the limitations of the method and the reproducibility of the cell distributions was gained in this manner. The gradients described here are representative examples of lymph and lymph node gradients.

Gradient Profiles of Lymph Cells During the Immune Response

EXPERIMENTAL

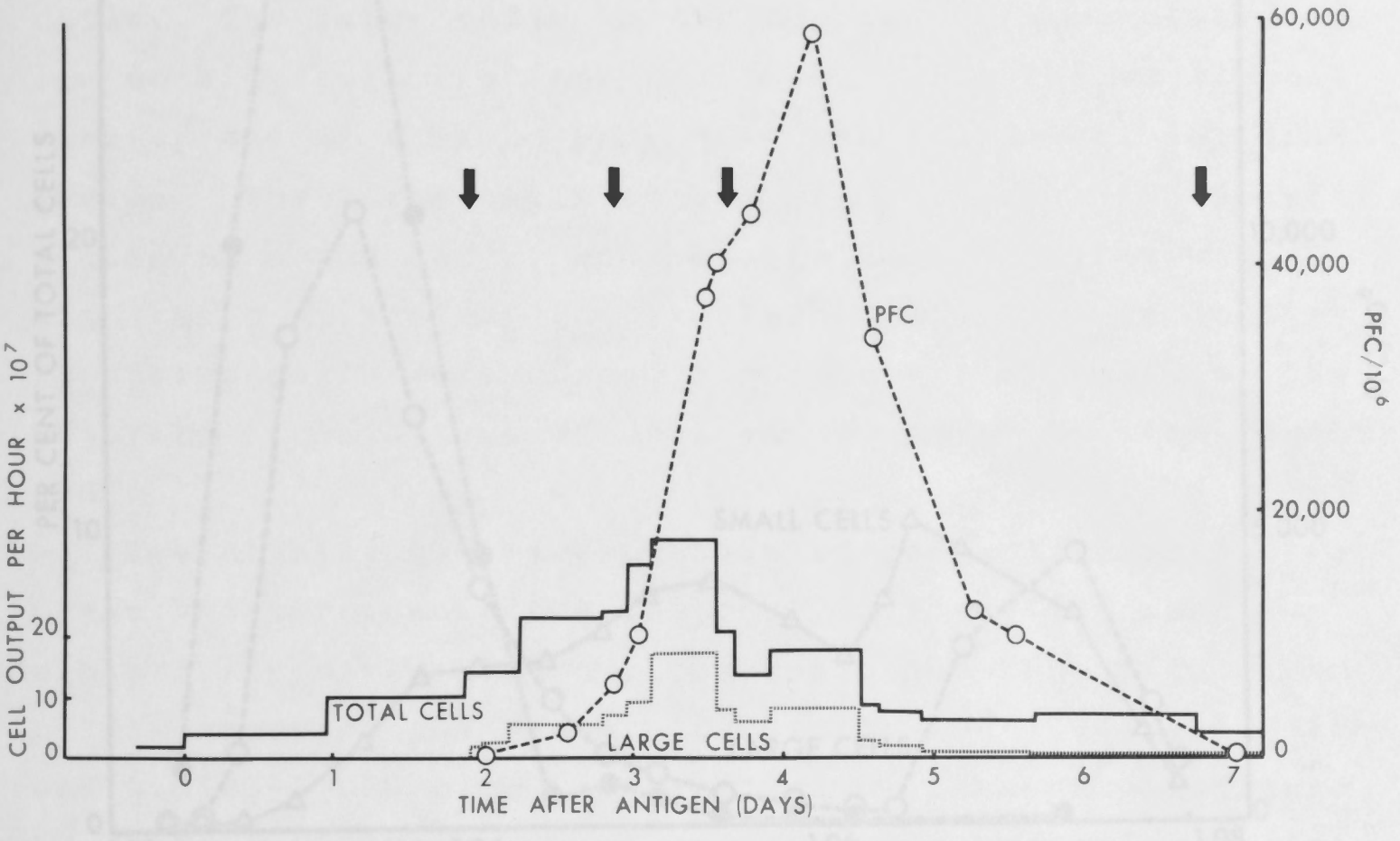
A sheep was given a secondary challenge of Salmonella in the lower leg and the cell output from the node, the production of antibody-forming cells and the proportion of large cells (volume >210 μ^3) in the cell population were measured throughout the response. Cells were obtained at four stages of the response and separated on density gradients. The cells were collected from the gradient in 15-18 equal fractions and each fraction was assayed for its density, the number of cells present, the proportion of large and small cells, the number of antibody-forming cells (PFC's) and the morphological characteristics of the cells as seen in Leishman smears, in the electron microscope and after staining with fluorescent antibody stain.

RESULTS

Figure IV-1 shows the cellular changes observed in the lymph leaving the popliteal node after antigenic stimulation. The response was similar to those described previously by Cunningham *et al* (1966). The output of cells increased from the second day after antigenic stimulation and reached a maximum between the third and fourth day. The output of large cells and PFC's also reached a maximum at this time. The cellular output then returned to the prestimulation level.

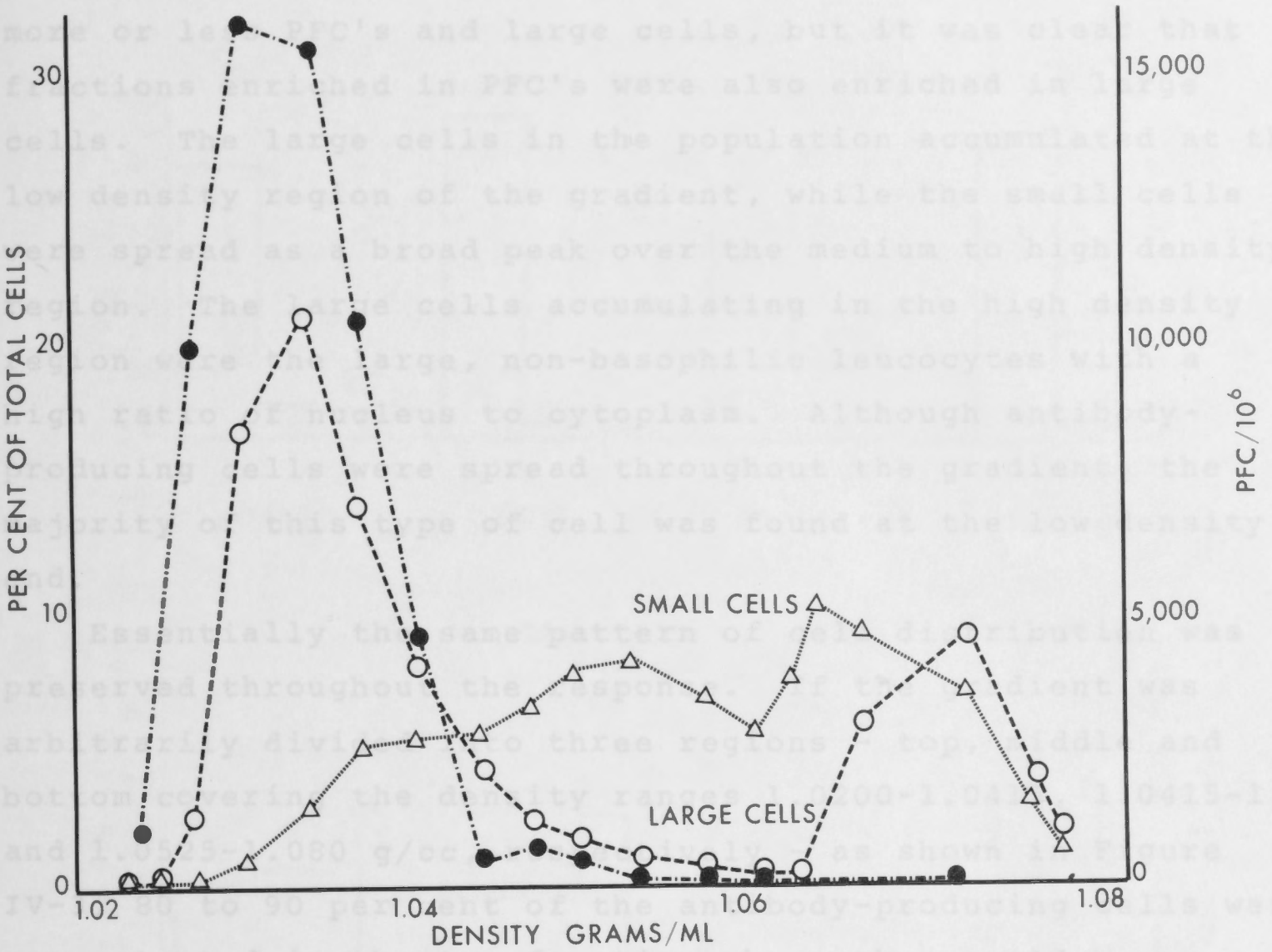
Figure IV-2 shows the patterns of the cell fractions obtained from Sample 2, collected over the period 71-73 hours after antigenic challenge. In this Figure, the distribution of large and small cells has been plotted as the percentage of the total population of cells found in each density level. Antibody-forming cells were plotted as the specific activity (PFC/10^6 cells) at each density level. The original cell suspension was made up of 6,579 PFC/10^6 cells and of the total cell population, 30.6 per cent were large cells. After separation on the density gradients, one fraction was recovered which contained 15,992 PFC/10^6 cells

FIGURE IV - 1



The cellular response in the efferent lymph of the popliteal lymph node after the injection of Salmonella organisms. Density gradient separations were carried out at the times indicated by the arrows.

FIGURE IV - 2



The density distribution of antibody forming cells in the lymph in relation to the distribution of large and small cells.

and 79.3 per cent large cells, while another fraction contained 55 PFC/10^6 cells and 1.2 per cent large cells. Between these two extremes, the other fractions contained more or less PFC's and large cells, but it was clear that fractions enriched in PFC's were also enriched in large cells. The large cells in the population accumulated at the low density region of the gradient, while the small cells were spread as a broad peak over the medium to high density region. The large cells accumulating in the high density region were the large, non-basophilic leucocytes with a high ratio of nucleus to cytoplasm. Although antibody-producing cells were spread throughout the gradient, the majority of this type of cell was found at the low density end.

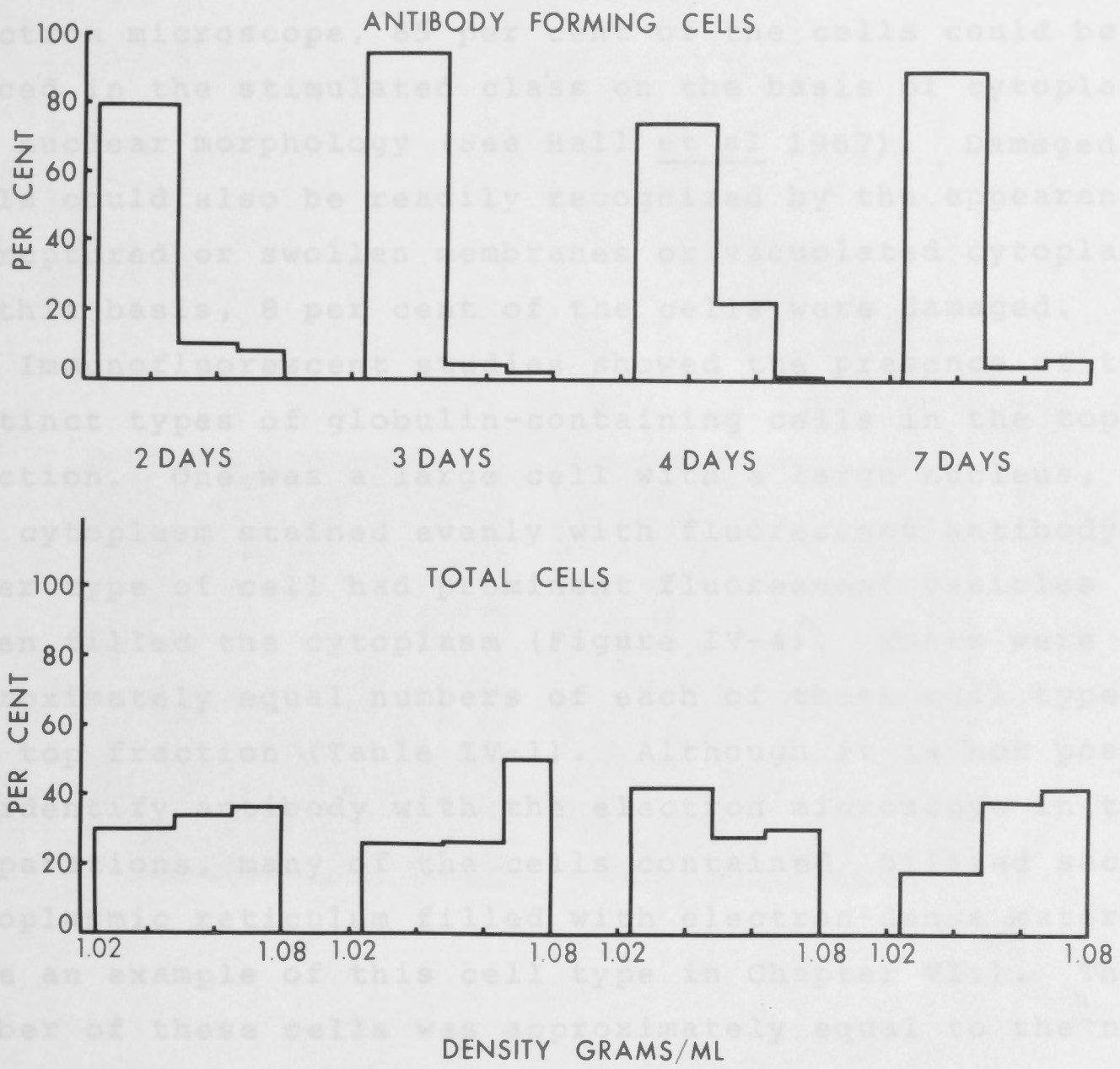
Essentially the same pattern of cell distribution was preserved throughout the response. If the gradient was arbitrarily divided into three regions - top, middle and bottom covering the density ranges 1.0200-1.0415, 1.0415-1.0525 and 1.0525-1.080 g/cc, respectively - as shown in Figure IV-3, 80 to 90 per cent of the antibody-producing cells were concentrated in the top fraction where they could be recovered free from the other lymph cells.

Morphological Characterization of the Antibody-Forming Cells Recovered from Lymph Density Gradients

EXPERIMENTAL

A sheep was challenged with a secondary injection of Salmonella and lymph cells near the peak of the immune response were subjected to the gradient separation procedure. The cells in an enriched fraction from the top of the gradient were examined using light, fluorescense and electron microscopy. A comparison was made between the numbers of cells forming plaques, the numbers of cells staining with fluorescent antibody, and the numbers having the appearance of transformed cells.

FIGURE IV - 3



The density distribution of antibody forming cells and total cells from the lymph at various stages of the immune response.

RESULTS

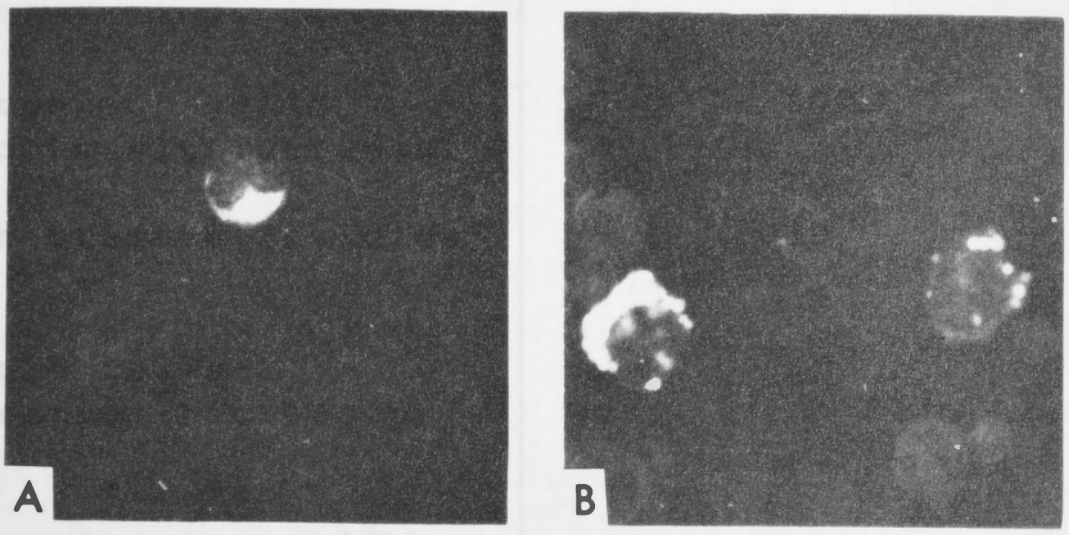
Table IV-1 shows the results of this study. Based on Leishman-stained smears, 70 per cent of the cells were classified as stimulated cells. When examined with the electron microscope, 85 per cent of the cells could be placed in the stimulated class on the basis of cytoplasmic and nuclear morphology (See Hall et al 1967). Damaged cells could also be readily recognized by the appearance of ruptured or swollen membranes or vacuolated cytoplasm. On this basis, 8 per cent of the cells were damaged.

Immunofluorescent studies showed the presence of two distinct types of globulin-containing cells in the top fraction. One was a large cell with a large nucleus, and its cytoplasm stained evenly with fluorescent antibody; the other type of cell had prominent fluorescent vesicles that often filled the cytoplasm (Figure IV-4). There were approximately equal numbers of each of these cell types in the top fraction (Table IV-1). Although it is not possible to identify antibody with the electron microscope in these preparations, many of the cells contained dilated sacs of endoplasmic reticulum filled with electron-dense material. (See an example of this cell type in Chapter VI.). The number of these cells was approximately equal to the number of cells with fluorescent vesicles.

The Density Distribution of Cells Obtained from the Medulla of Stimulated Lymph Nodes

It was apparent from preliminary investigations that the proportion of antibody-forming cells in the node was relatively low throughout the whole of the response when compared with the lymph. In an attempt to increase the proportion of antibody-forming cells placed on the gradients, lymph nodes were separated into cortex and medulla, sliced into thin sheets and the cells separated by dissection. The following experiments were carried out with cells obtained from the medulla of stimulated lymph nodes in this way.

FIGURE IV - 4



Examples of the two types of fluorescent cells obtained from the low density region of the lymph gradient. The cells were stained with fluorescent anti-sheep globulin.

Magnification x 500.

TABLE IV-1

Characteristic Examined	No. Positive/No. Counted	%
Stimulated cells (Light Microscopy)	770/1100	70
Stimulated cells (Electron Microscopy)	196/231	85
Fluorescent cells (Smooth Fluorescence)	40/1000	4
(With Stained Vesicles)	30/1000	3 — 7
Damaged cells (Electron Microscopy)	18/231	8
Plaque forming cell activity/10^6 cells	76,500	8

Characteristics of the cells isolated from the low density region of a lymph cell gradient.

Although somewhat variable results were obtained in different experiments, the proportion of antibody-forming cells was significantly higher from isolated fragments of medulla than from the whole node or from the cortex (Table IV-2).

Figure IV-5 shows the density distribution of cells obtained from the medulla of the popliteal lymph node four days after a secondary challenge. The distribution of large and small cells was similar to that seen in the case of lymph-borne cells, although the peak of large cells at the low density end of the gradient was not as distinct as that found when lymph cells were separated (Figure IV-2) and there was a considerable accumulation of small cells at the high density end of the gradient. The major difference between the lymph node and lymph cell distributions was that the antibody-forming cells from the lymph node were more evenly spread throughout the whole of the gradient. When the gradient for the lymph node cells was divided into top, middle and bottom fractions and the total number of antibody-forming cells in each was plotted (Figure IV-6), 74 per cent of the antibody-forming cells were present in the middle and bottom fractions.

Morphological Features of Cells from the Medulla of Lymph Nodes

Cells from each of the fractions collected from the gradient (Figure IV-5) were examined after staining with fluorescent anti-sheep globulin.

There were some differences in the morphology of fluorescent cells seen in the lymph node gradient compared with those observed when efferent lymph cells were fractionated in the same way. Cells showing both vesicular and smooth cytoplasmic fluorescence (Figure IV-7) were confined predominantly to the top fraction. However, others having the characteristic morphology of plasma cells (Figure IV-7) were also present in this fraction. The plasma cells were most abundant in the middle fraction where they comprised approximately 50 per cent of the fluorescent population. Cells of this type are rare in efferent lymph (Hall *et al* 1967).

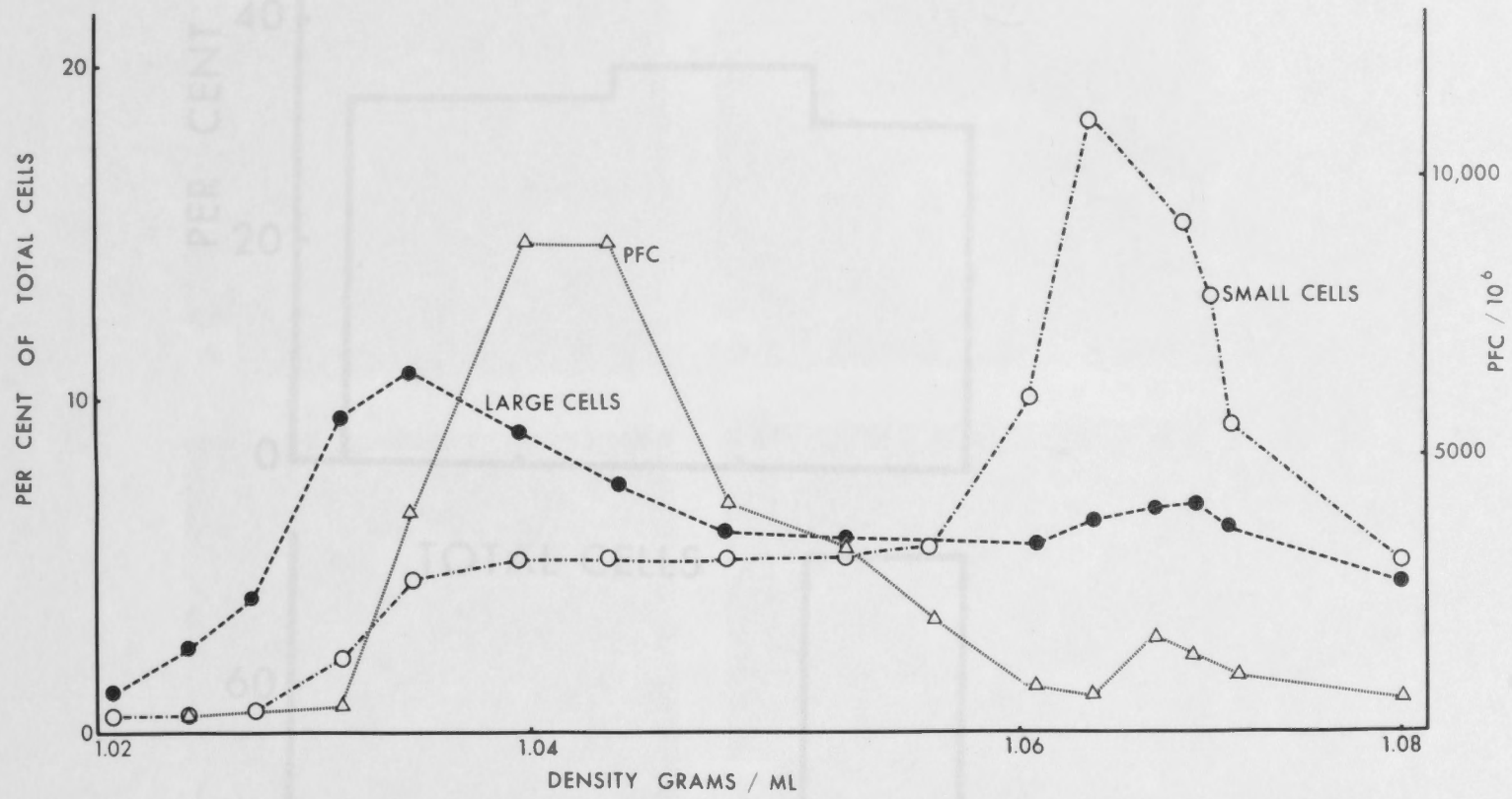
TABLE IV-2

Days after immunization	PFC per 10^6 cells		
	Cortex	Medulla	Ratio*
4	1464	6699	4.6
4	18	44	2.4
4	12	149	12.4
4	36	93	2.6
4	27	70	2.6
5	254	2059	8.1
5	325	4392	13.5
6	43	104	2.4

$$* \quad \text{Ratio} = \frac{\text{PFC}/10^6 \text{ Medulla cells}}{\text{PFC}/10^6 \text{ Cortex cells}}$$

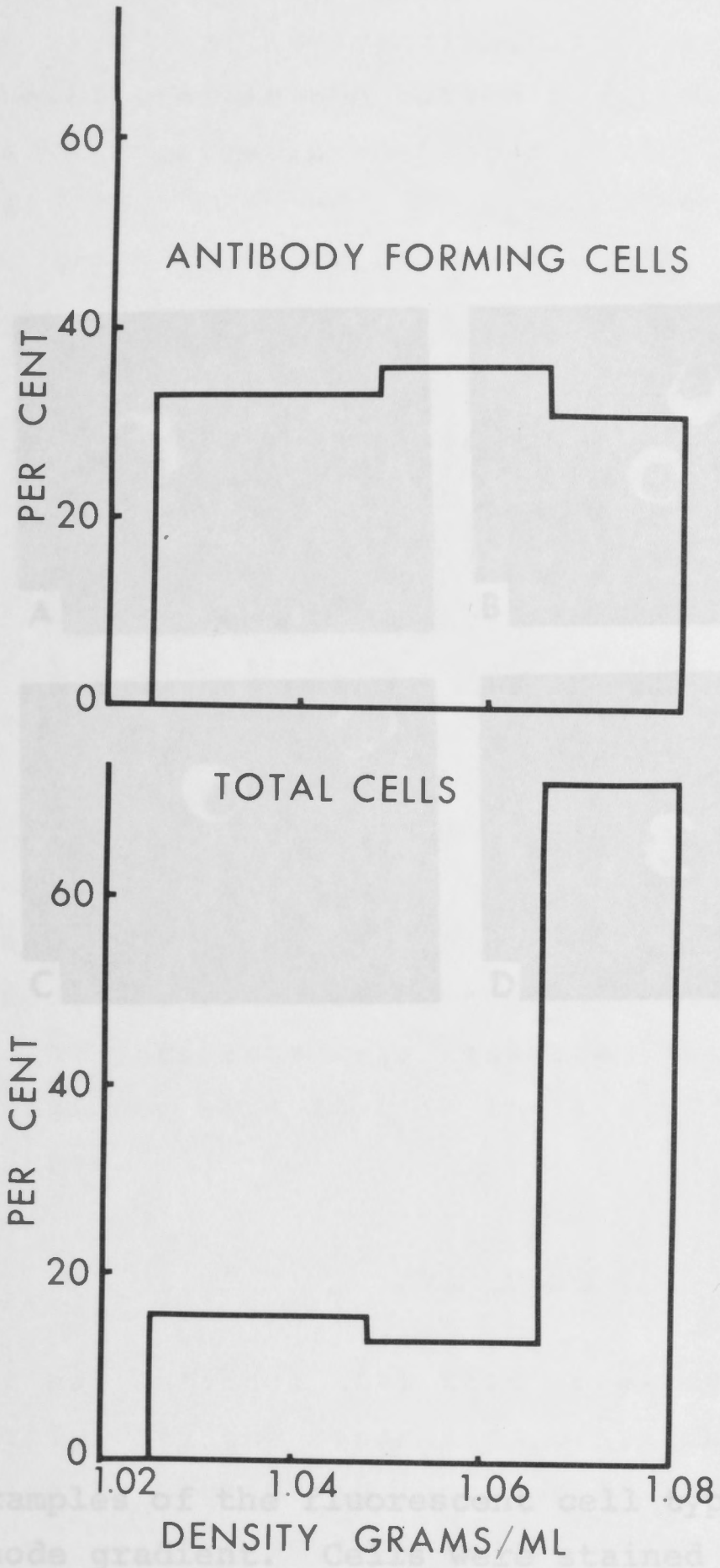
A comparison of the PFC activity in the cortex and medulla of popliteal lymph nodes.

FIGURE IV - 5

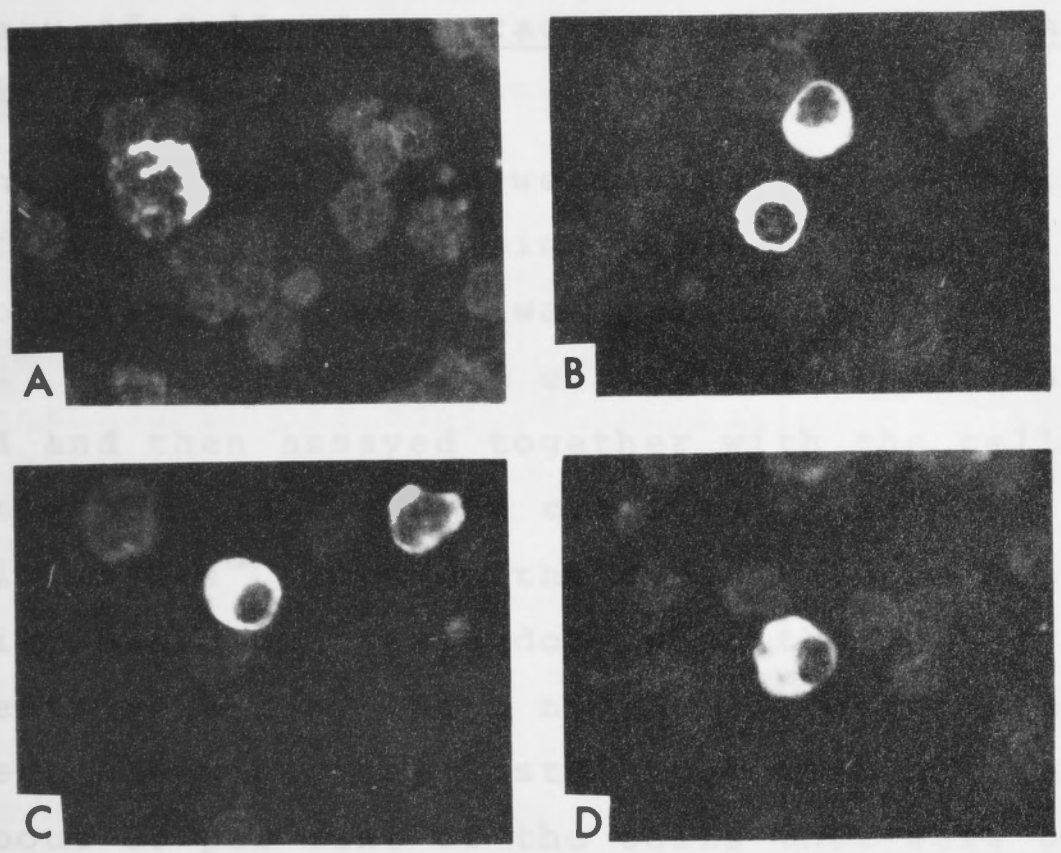


The density distribution of antibody forming cells in the lymph node in relation to the distribution of large and small cells.

FIGURE IV - 6



The density distribution of antibody forming cells and total cells from the medulla of a lymph node 4 days after antigenic challenge.



Examples of the fluorescent cell types obtained from the lymph node gradient. Cells were stained with fluorescent anti-sheep globulin. The cell with fluorescent vesicles was obtained from the top fraction and the other examples were obtained from the middle fraction.

Magnification x 500.

Cells obtained by teasing apart the lymph node had a low viability and only about 70 per cent of these cells appeared viable when tested for their capacity to exclude trypan blue. After fractionation, the majority of the dead cells collected in the bottom fraction; more than 90 per cent of the cells in the upper part of the gradient were viable.

Recovery of Cells from Gradients and Loss of Activity in Albumin

The cell populations were assayed for their ability to produce plaques before being subjected to the albumin gradients. A second sample of cells was maintained in 35 per cent albumin at 0-4°C for the duration of the fractionation and collection period and then assayed together with the cells harvested from the gradient. Lymph cells maintained in 35 per cent albumin at 0-4°C lost on the average 20 per cent of their activity based on assays done when the four lymph cell gradients were run. When node cells were maintained in 35 per cent albumin, they lost 61 per cent of their activity.

About 85 per cent of the cells that were originally placed on gradients were recovered again in the fractions. The remainder were lost in the fractionation and washing procedures.

Discussion

It was intended that this investigation be concerned primarily with the separation of antibody-forming cells from other cells present in lymph or in lymph nodes. The first point to be made in this regard is that a considerable enrichment of antigen-stimulated cells can be achieved by the biological fractionation that is inherent in the collection of efferent lymph cells as against the harvesting of cells from the whole lymph node. In response to stimulation with Salmonella antigens, the activity of antibody-producing cells in efferent lymph can be as high as 60,000 PFC/10^6 total cells. The highest activity reported by Hummler, Harris,

Tomassini, Hechtel and Farber (1966) in efferent popliteal lymph of rabbits was 48 PFC/10^6 cells. Haskill, Legge and Shortman obtained a maximum of 600 PFC/10^6 cells in rat thoracic duct lymph after immunization. Values approximating 6,000/10^6 cells are an upper limit to the PFC activity obtained from lymph node cells, and values of this magnitude are only obtained when the medulla is physically separated from the cortex, as the cortex contains a large proportion of the unreactive cells in the lymph node. The physical separation achieved by density gradient centrifugation can further increase the PFC activity by a factor of 2 - 5 for cells from both lymph and lymph nodes.

Density gradient analysis showed that lymph-borne PFC's consisted predominantly of low density cells; in these gradients 80-90 per cent of the antibody-forming cells collected in the region ranging in density from 1.020-1.042 g/cc. Moreover, the density distribution of these cells did not change significantly throughout the response. In relation to the results described in Chapter III where it was shown that the antibody-forming cells release (and presumably contain) more antibody towards the end of the response, it must be concluded that these metabolic activities have little effect on the density of the cells.

The antibody-forming cells appearing in lymph are a sample of similar cells produced in the stimulated node, but the relative proportions of high and low density cells are different. In contrast to the lymph where 80-90 per cent of the antibody-forming cells were distributed in the top fraction, only 30 per cent of antibody-forming cells from the node were present in the top fraction. Mature plasma cells are rarely found in efferent lymph (Hall et al 1967) and cells with the characteristic morphology of mature plasma cells which are present in large numbers in lymph nodes collected predominantly in the middle density region of the gradients. It would appear, therefore, that the different density distribution of PFC's in lymph as compared with the lymph node could be accounted for if the majority of the plasma cells remained in the node and that low density, antibody-forming cells act as part of a much more mobile cell

population that leave the node and pass readily into the efferent lymph stream.

Enriched fractions of antibody-forming cells still contained a high proportion of cells that did not form plaques or stain with fluorescent antiglobulin antiserum. 70-85 per cent of the cells were classified as stimulated on morphological grounds but less than 10 per cent were shown to either contain or release antibody. There are several possible reasons that may explain this finding. In the first place, the sensitivity of either the plaque assay or the fluorescent antibody staining procedure is not known. Cells might contain small amounts of antibody which these assays could not detect. Alternatively, nothing can be said concerning the immediate past history or the future of the stimulated cells. They may have recently released antibody or they may continue to differentiate and commence synthesizing antibody at a later date and so become positive in a plaque assay. Additionally, boiled Salmonella muenchen is by no means a pure, single antigen. Some of the stimulated cells would almost certainly be involved in producing antibody against denatured flagellar proteins or antigens other than the lipopolysaccharides whichwere being tested for in the assay. A further possibility might be that these stimulated cells were reacting to antigen but did not develop into antibody-forming cells. This is the type of cell considered by some to be a component of immune responses and to originate in the thymus. (Claman, Chaperon and Triplett, 1966; Davies, Marchant and Elliot, 1967; Mitchell and Miller, 1968; Nossal, Cunningham, Mitchell and Miller, 1968). In view of the results described in Chapter V using the single, pure antigen horse-radish peroxidase, it seems probable that most if not all of the stimulated cells present in lymph are involved directly in antibody synthesis.

Haskill, Legge and Shortman (1969) described the fractionation of antibody-forming cells from rat thoracic duct lymph on albumin gradients. Following intravenous and intraperitoneal doses of sheep red cells, thoracic duct lymph appeared to contain a significant increase in the proportion

of low density PFC's compared with spleen or lymph nodes. However, they found that the majority of lymph antibody-forming cells were still in the more dense region of the gradient. Rat antibody-forming cells isolated from the thoracic duct were reported to be very fragile and had a half-life of about 1.5 hours after they were collected. In contrast, the cells from the lymph of the sheep were more amenable to the manipulation procedures than were the cells teased from fixed lymphoid tissue.

Summary

1. Antigen-stimulated cells leaving the lymph node by way of the efferent lymph are predominantly low density cells, 80-90 per cent of the antibody-forming cells accumulating in the density region 1.020 to 1.042 g/cc.

2. Antibody-forming cells isolated from a lymph node cover a wider density range; some 70 per cent of these cells have a density greater than 1.042 g/cc.

3. Morphological analysis indicated that the low density cells produced in the node can readily leave the node via the efferent lymph, while the higher density cells which include the plasma cells, tend to remain associated with the lymph node.

4. Density gradient separation provides an effective means for separating antigen-stimulated cells from the other cells in efferent lymph.

CHAPTER V

CELL PROLIFERATION AND DIFFERENTIATION DURING THE IMMUNE RESPONSE

Cell Proliferation and Differentiation During the Immune Response

If the proposition is accepted that an immune response results from the stimulation of immunologically competent cells (lymphocytes) by antigen and that these cells subsequently differentiate, proliferate and produce specific antibody, it should be possible to trace this sequence of events by studying a single cell. Experimentally, this has never been achieved and probably never will be because a single cell, so isolated to enable these observations to be made, would be removed from various environmental influences that would determine its life-history in the intact, living animal. However, even though such a study is unlikely to be successful with single cells, an idea of the sequence of cellular events that accompany an antigenic stimulus can be obtained by following the biochemical activities and ultrastructural characteristics of a population that is made up of differentiating cells which are reacting to antigen. Such a cell population can be obtained from the efferent lymph of the popliteal node.

The experiments described in this chapter were designed to examine the cell populations in efferent lymph at different times after antigenic challenge to see whether the populations as a whole became more differentiated as the response progressed. The specific properties of individual cells were then correlated with those of the general population.

Results

Changes in the Properties of the Efferent Lymph Cell Population at Different Stages of the Immune Response

EXPERIMENTAL

Sheep were challenged in the lower leg with primary injections of Salmonella or secondary injections of horse-radish peroxidase. During these responses the following

measurements were made on the efferent lymph:

1. The cell numbers and lymph flow rates.
2. The proportion of blast cells from Leishman stained smears.
3. The proportion of antibody-forming cells (PFC's were assayed during the Salmonella responses; cells containing antibody to horse-radish peroxidase were stained after complexing antibody with enzyme and substrate and examined by light microscopy under an oil immersion lens).
4. The amount of ^{3}H-thymidine and ^{3}H-uridine incorporated into acid precipitable material following a 1 hour incubation of 5×10^{5} cells - 5×10^{6} cells in 1 ml of Eagle's medium at 37° C. The medium contained 10 per cent foetal calf serum and labelled nucleic acid precursor at 1 or 2µCi/ml.
5. The lactic dehydrogenase (LDH) content of the cell populations.
6. The antibody content of the lymph.

RESULTS

Table V-1 shows the results of a Salmonella response. The uptake of ^{3}H-thymidine/10^{6} cells increased more rapidly during the early stages of the response than did the numbers of PFC's and blast cells. One explanation of this result was that the early cells, although not yet differentiated enough to have detectable basophilic cytoplasm or to be releasing sufficient antibody to produce a plaque had already begun to synthesize DNA and were incorporating ^{3}H-thymidine.

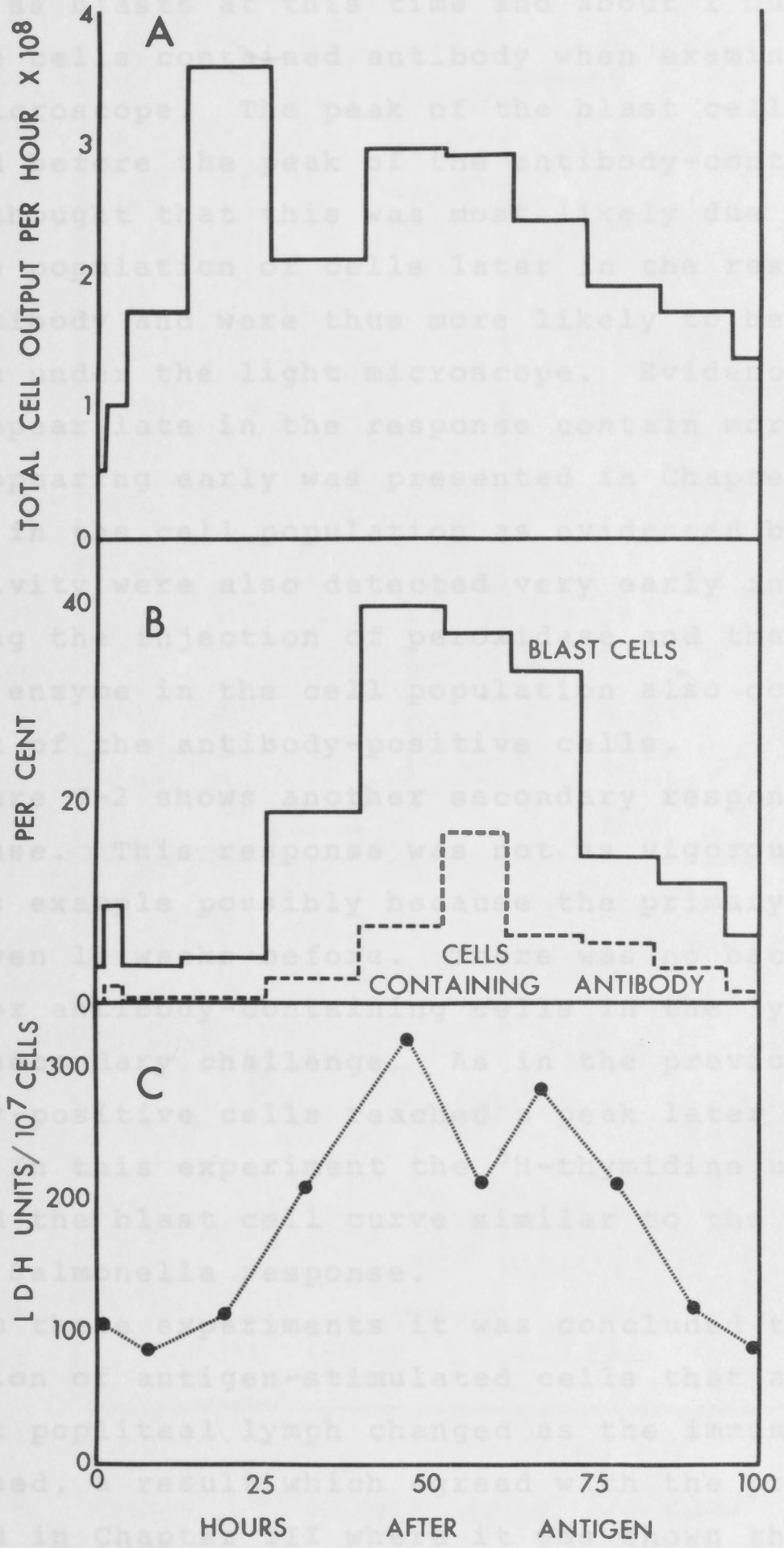
Figure V-1 shows a secondary response to peroxidase. This secondary challenge was given 6 weeks after a primary challenge with antigen and incomplete Freund's adjuvant. The background level of blasts and antibody-containing cells in the lymph early in the response was due to the continuing response from the chronic granuloma caused by the primary injection. The secondary response to this antigen reached a maximum before 60 hours. In this particular experiment the antibody-containing cells constituted about 17 per cent

TABLE V-1

Time	Blasts %	PFC/10^6	^{3}H-thymidine cpm/10^6	^{3}H-uridine cpm/10^6
Before antigen	< 1	0	109 ± 6.6	134 ± 17.5
Day 1	< 1	0	91 ± 5.5	228 ± 55.8
Day 2	1.0	0	204 ± 14.8	168 ± 14.8
Day 3	6.8	430	3,920 ± 240.8	408 ± 18.9
Day 4	31.3	6,500	6,327 ± 605.3	705 ± 36.8
Day 5	20.1	939	1,404 ± 125.9	76 ± 13.0
Day 6	2.6	262	395 ± 139.3	227 ± 21.2

The incorporation of ^{3}H-thymidine and ^{3}H-uridine into TCA precipitable material after 1 hour at 37°C during a primary response to Salmonella. Mean values of triplicate samples are expressed ± the standard deviations.

FIGURE V - 1



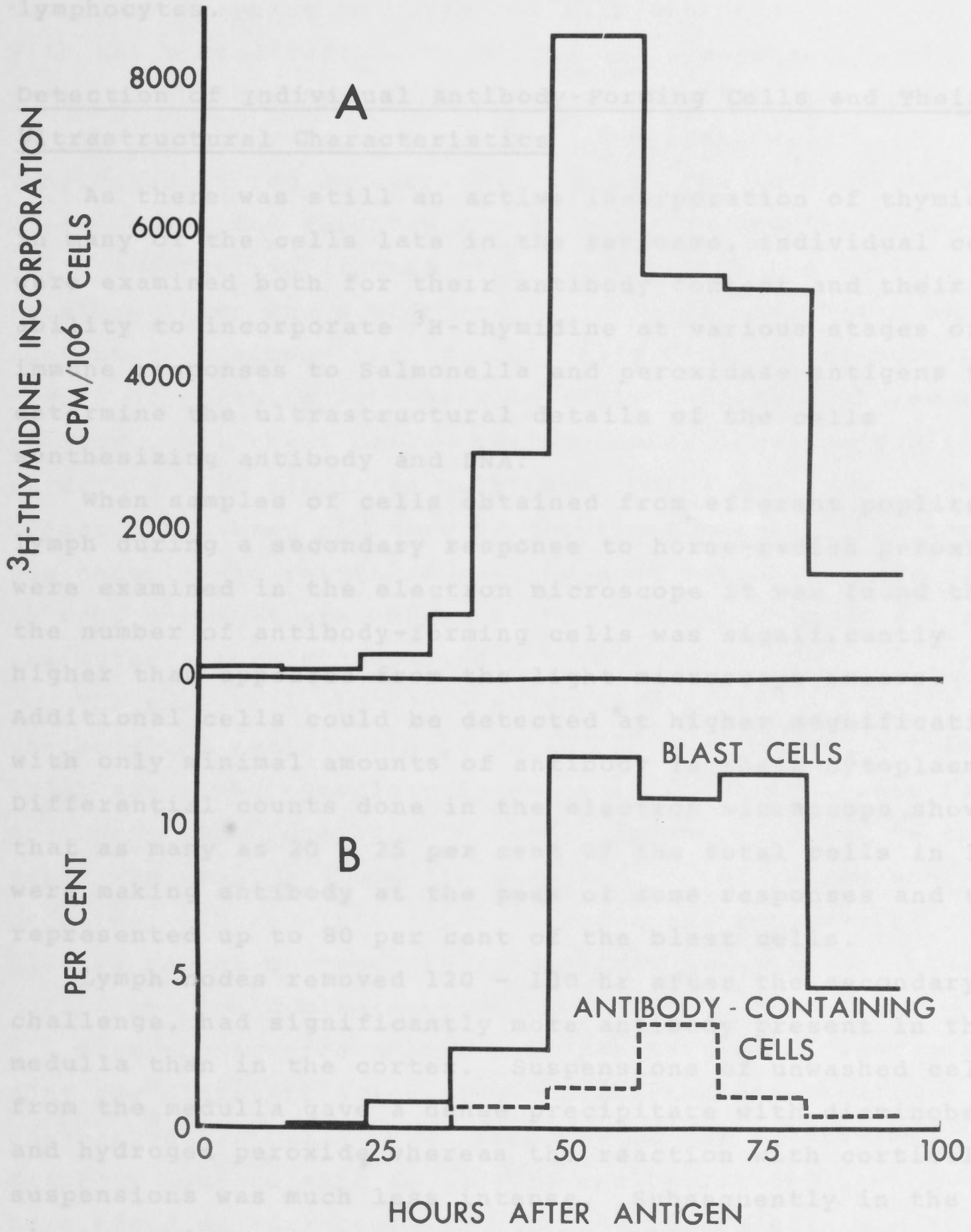
A secondary immune response to horse-radish peroxidase.

of the total population at the height of the response. Approximately 37 per cent of the cells in the lymph were classed as blasts at this time and about 1 out of every 2 of these cells contained antibody when examined with the light microscope. The peak of the blast cell response occurred before the peak of the antibody-containing cells and it was thought that this was most likely due to the fact that the population of cells later in the response contained more antibody and were thus more likely to be scored as positive under the light microscope. Evidence that the PFC's which appear late in the response contain more antibody than those appearing early was presented in Chapter III. Metabolic changes in the cell population as evidenced by increasing LDH activity were also detected very early in the response following the injection of peroxidase and the peak activity of this enzyme in the cell population also occurred before the peak of the antibody-positive cells.

Figure V-2 shows another secondary response to peroxidase. This response was not as vigorous as the previous example possibly because the primary challenge had been given 10 weeks before. There was no background of blasts or antibody-containing cells in the lymph at the time of the secondary challenge. As in the previous example the antibody-positive cells reached a peak later than the blast cells. In this experiment the ^{3}H-thymidine uptake curve preceded the blast cell curve similar to the result described for the Salmonella response.

From these experiments it was concluded that the population of antigen-stimulated cells that appeared in efferent popliteal lymph changed as the immune response progressed, a result which agreed with the previous findings reported in Chapter III where it was shown that the amount of antibody contained in cells and their reaction in the indirect plaque assay also changed as the response progressed. In general terms, populations of cells early in the response contained a high proportion of cells which incorporated ^{3}H-thymidine and contained small quantities of antibody. Many of these cells were not large blast cells.

FIGURE V - 2



A secondary immune response to horse-radish peroxidase.

The later cell populations featured cells which contained larger quantities of antibody and which varied in size from large blast cells to cells not much larger than lymphocytes.

Detection of Individual Antibody-Forming Cells and Their Ultrastructural Characteristics

As there was still an active incorporation of thymidine in many of the cells late in the response, individual cells were examined both for their antibody content and their ability to incorporate ^{3}H-thymidine at various stages of immune responses to Salmonella and peroxidase antigens to determine the ultrastructural details of the cells synthesizing antibody and DNA.

When samples of cells obtained from efferent popliteal lymph during a secondary response to horse-radish peroxidase were examined in the electron microscope it was found that the number of antibody-forming cells was significantly higher than appeared from the light microscope smears. Additional cells could be detected at higher magnification with only minimal amounts of antibody in their cytoplasm. Differential counts done in the electron microscope showed that as many as 20 - 25 per cent of the total cells in lymph were making antibody at the peak of some responses and this represented up to 80 per cent of the blast cells.

Lymph nodes removed 120 - 130 hr after the secondary challenge, had significantly more antibody present in the medulla than in the cortex. Suspensions of unwashed cells from the medulla gave a dense precipitate with diaminobenzidine and hydrogen peroxide whereas the reaction with cortical cell suspensions was much less intense. Subsequently in the electron microscope, mature plasma cells containing antibody were found in considerable numbers in medullary cell suspensions, but were less numerous in samples of cells from the cortex. Some blast cells with a similar ultrastructure to the antibody-forming cells found in the lymph earlier in the response were detected in both the cortical and medullary cell suspensions but they did not contain antibody at this stage.

It seemed profitless to attempt to categorize the antibody-forming cells in lymph other than in fairly general terms. At the outset it can be stated that during the secondary response to horse-radish peroxidase, no cells with the ultrastructure of plasma cells appeared in the lymph, even though this type of cell was abundant in the lymph node.

The antibody-forming cells in the lymph will be considered in two categories; firstly, the large transforming blast cells, and secondly the smaller lymphocytes. It is as well to establish at this point that within the first category there was a wide variety of cells in which the content of antibody varied from cells with little more than groups of positive ribosomes to cells replete with reaction product. All these cells however had an immature nuclear structure with dispersed marginated chromatin, one or more nucleoli and many clusters of polyribosomes in the cytoplasm. The endoplasmic reticulum was the most variable element, for in some cells it was quite rudimentary while in others it was ostentatiously developed.

The smaller lymphocytes which were found to contain antibody had nuclei with condensed chromatin, and a relatively small amount of cytoplasm in which the ribosomes were for the most part unaggregated. The endoplasmic reticulum in these cells was small and usually amounted to only a few short profiles. These distinctions between the two general cell classes cannot be regarded as absolute however, as intermediate cell types could always be found.

From the studies on the cell populations it was concluded that the differentiation sequence started with small, non-basophilic cells which probably contained very little antibody and proceded to larger, basophilic cells with more abundant intracellular antibody accumulations. Figures V-3 to V-8 show examples of lymph cells which can be considered to be representative of this sequence of differentiation.

Localization of antibody within cells. (a) Lymph cells. Antibody was identified in positive cells in the cytoplasm

FIGURE V-3

Lymph cells containing antibody obtained during an immune response to peroxidase. Phase-contrast micrograph. Magnification X 1600.

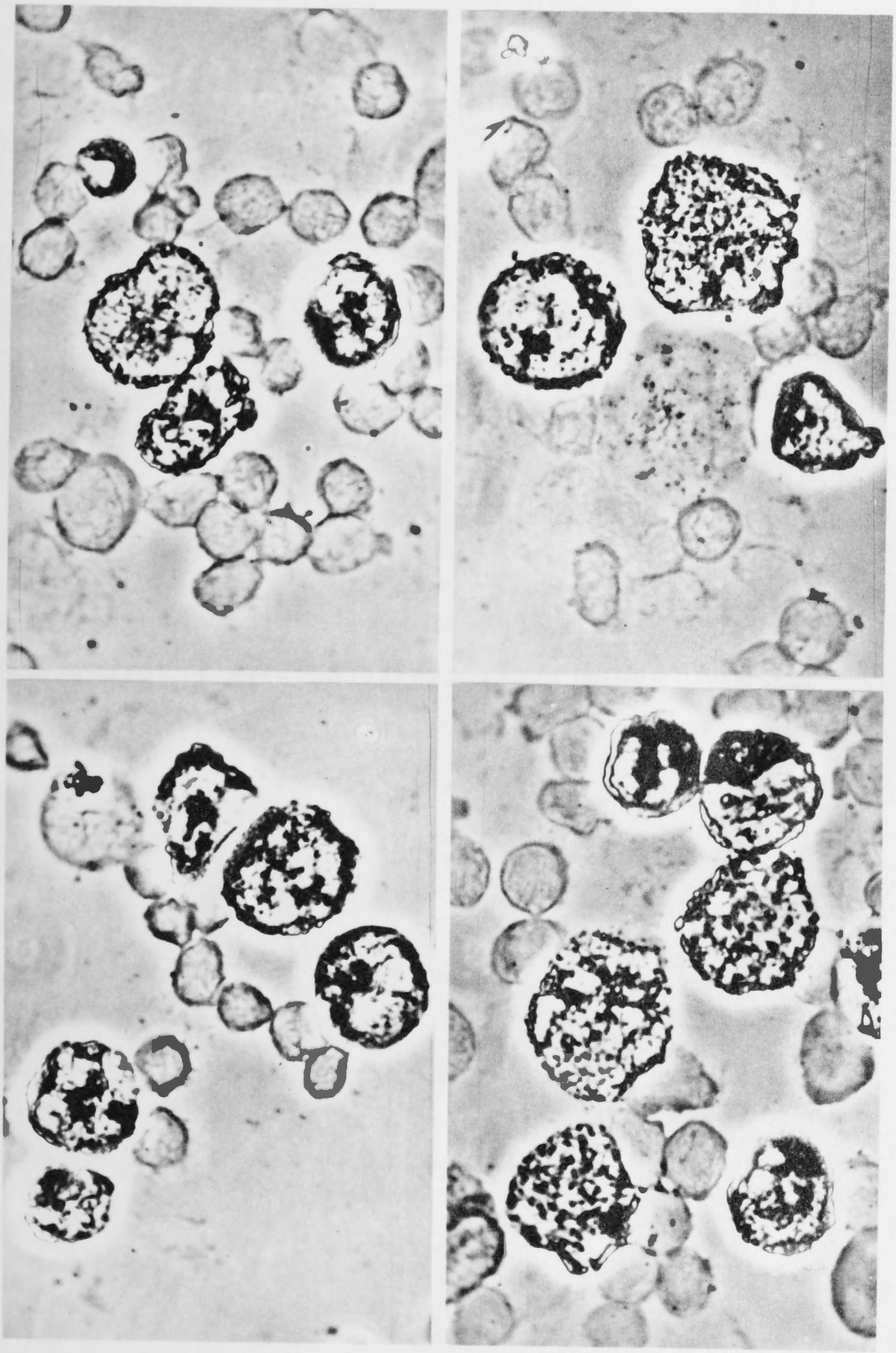


FIGURE V-4

Electron micrographs of lymphocytes obtained from efferent lymph 30 hours after challenge with horse-radish peroxidase. The reaction product is associated with the perinuclear space and the ergastoplasm.

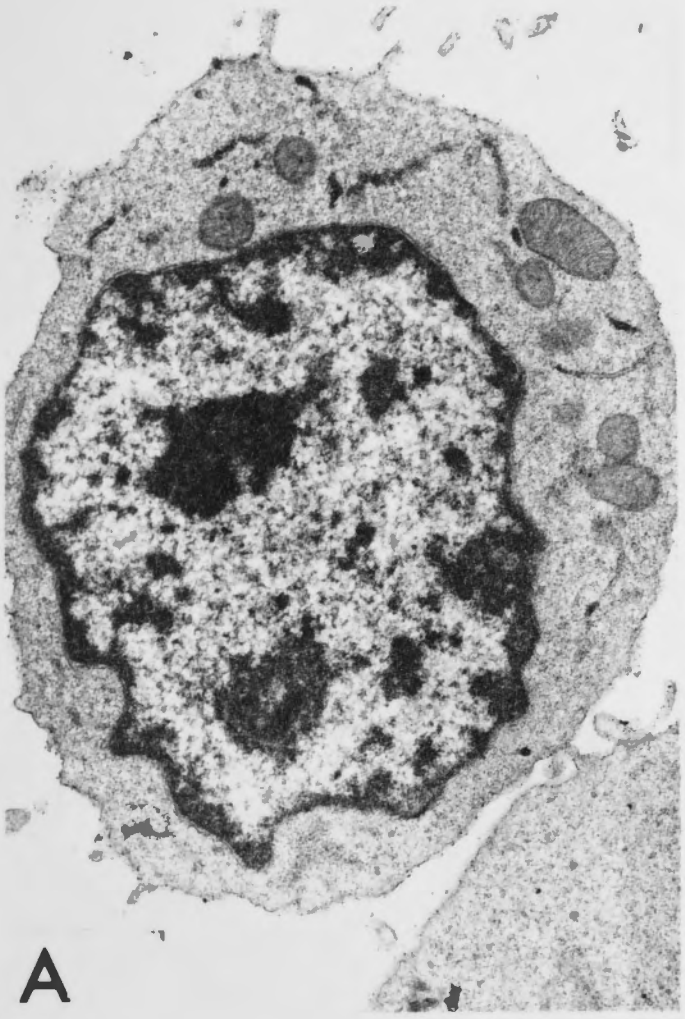
Magnifications

A X 9,600

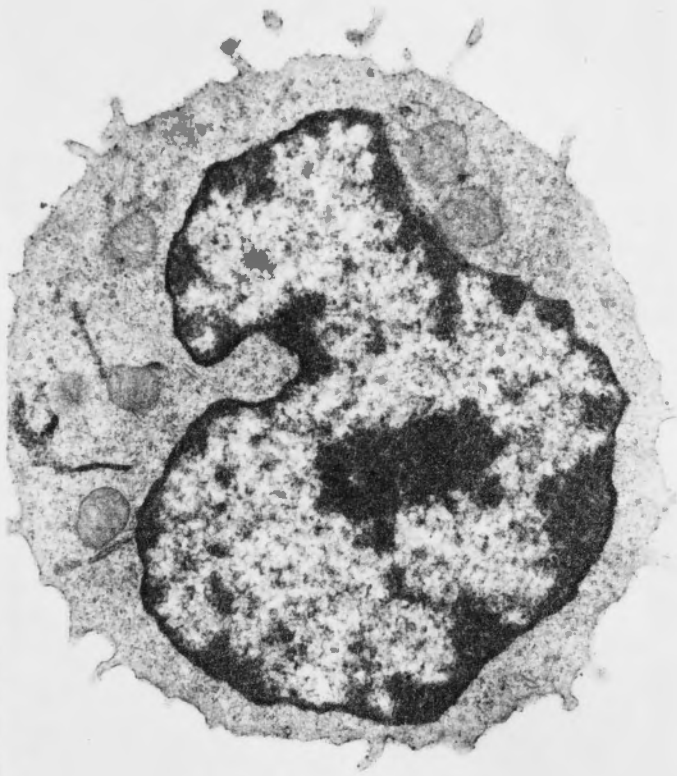
B X 9,200

C X 9,600

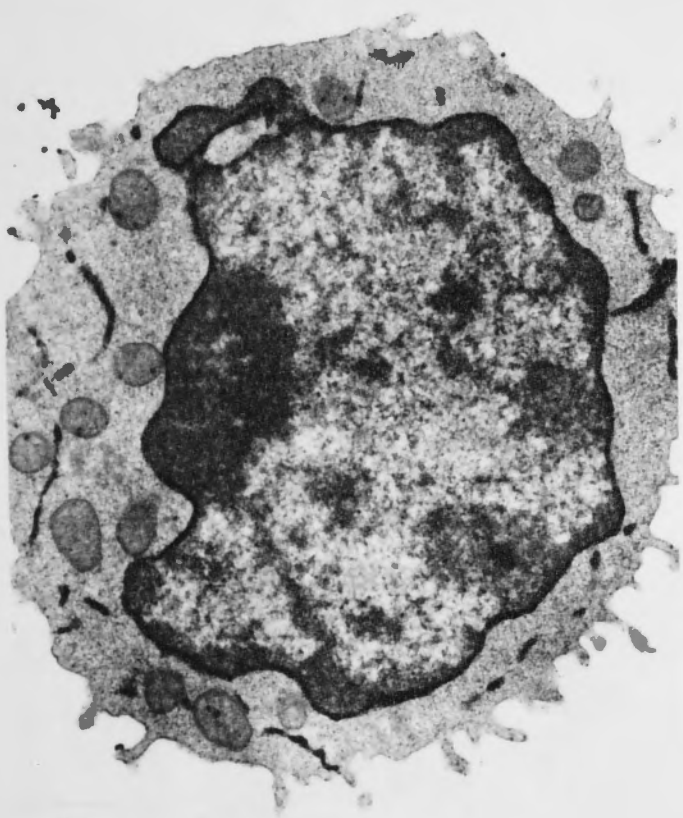
D X 9,600



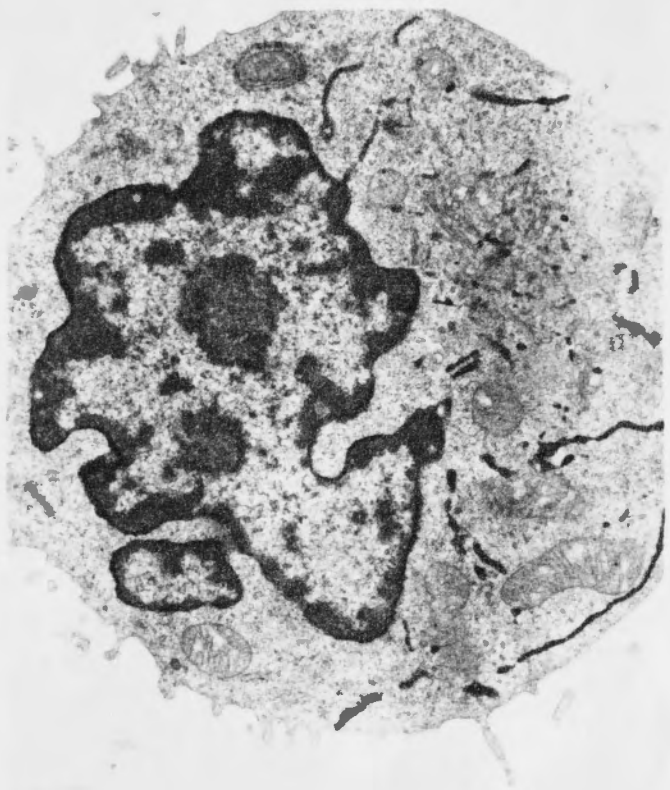
A



B



C



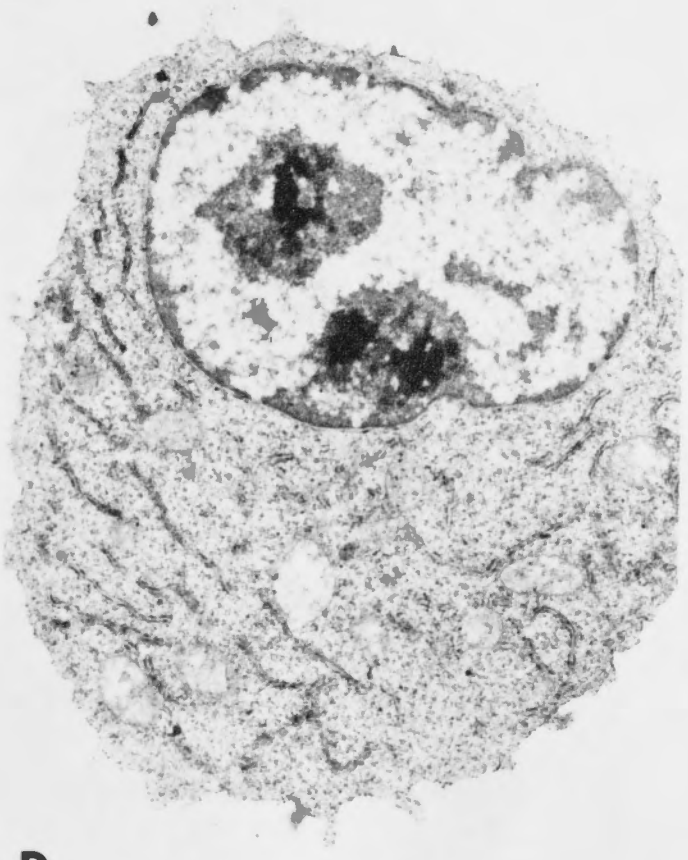
D

FIGURE V-5

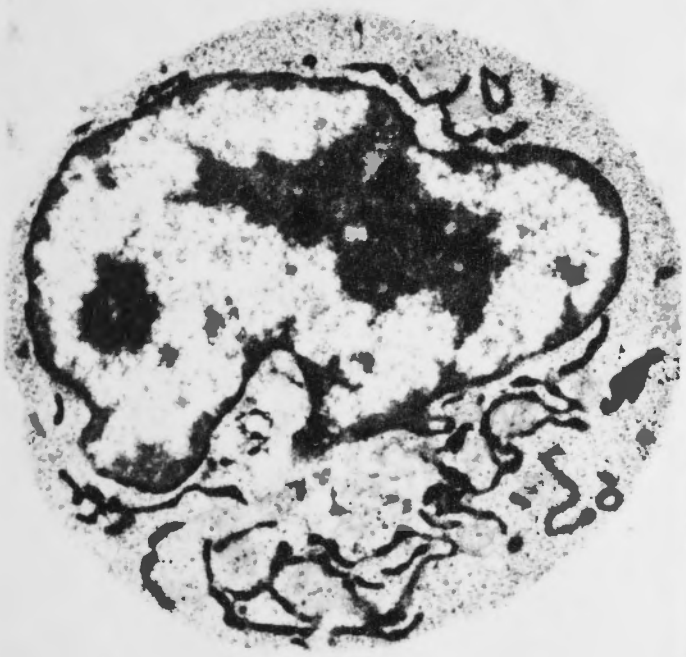
Electron micrographs of lymph cells with antibody to peroxidase associated with the nucleolus.

Magnifications:
A X 10,500
B X 10,900
C X 10,000
D X 64,600

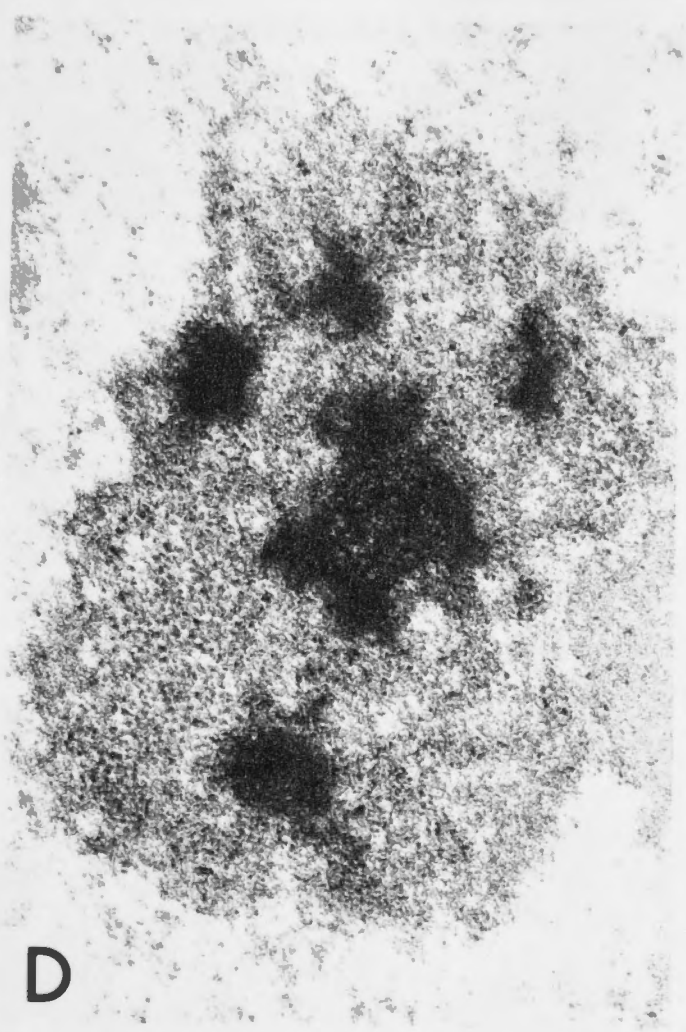
A



B



C

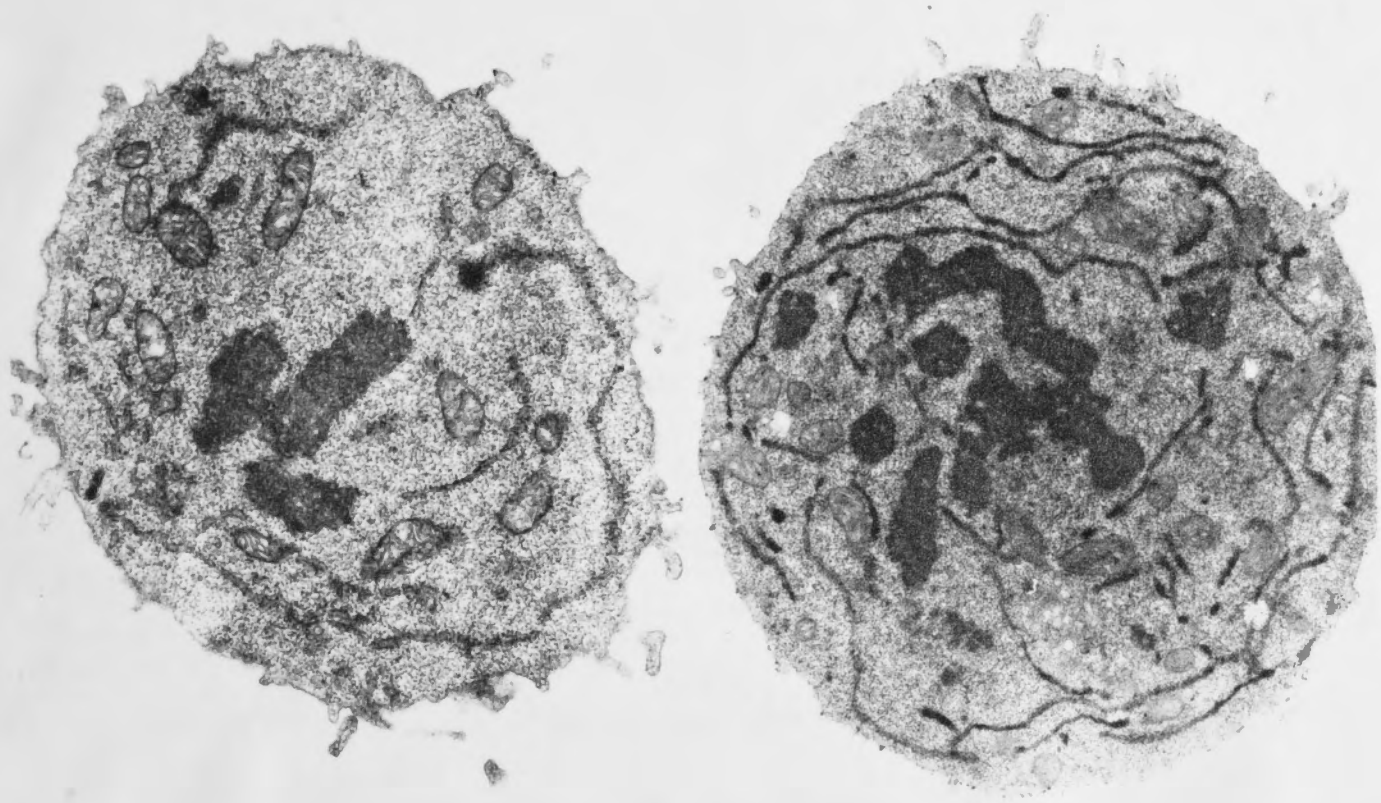


D

FIGURE V-6

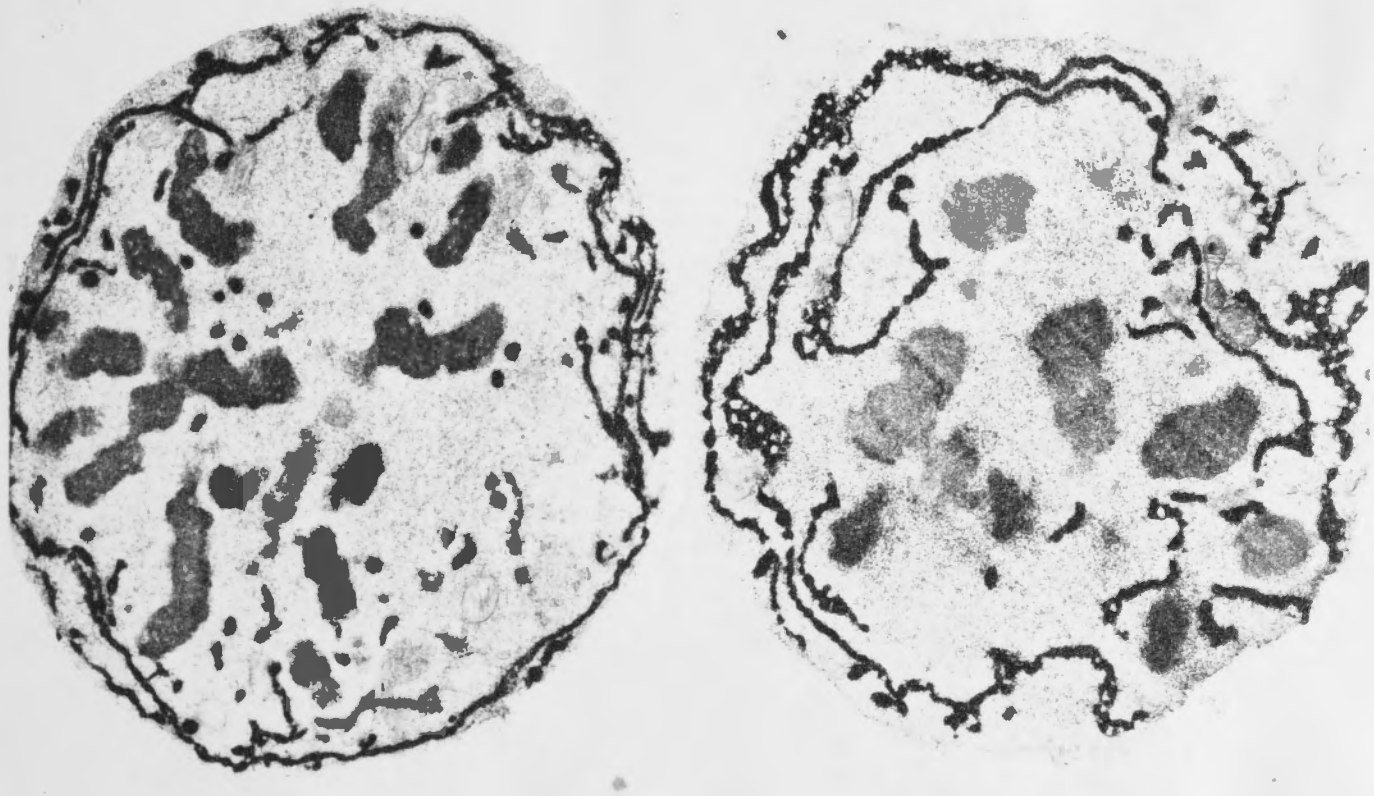
Electron micrographs of dividing lymph cells containing antibody to peroxidase. Most of the antibody is associated with the ergastoplasm.

Magnifications:
A X 10,000
B X 10,000
C X 8,300
D X 10,500



A

B



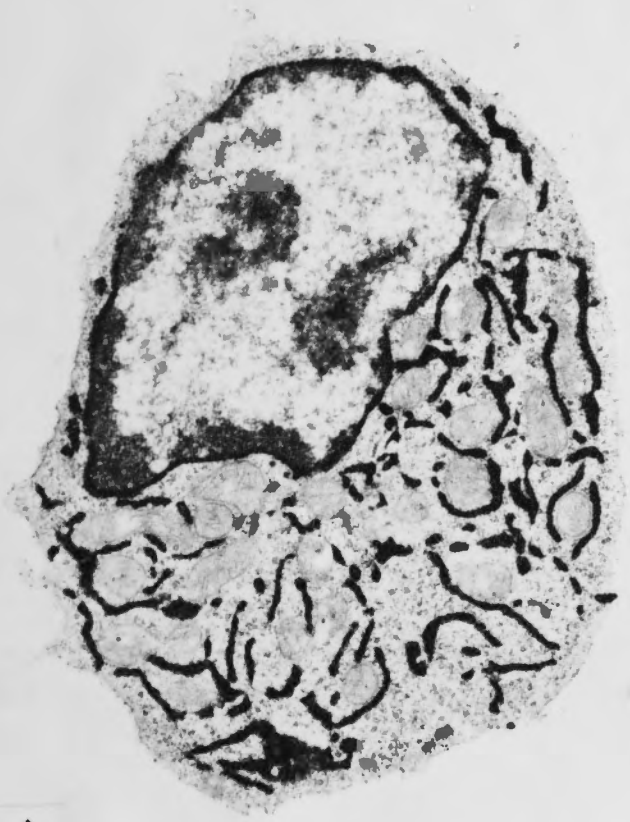
C

D

FIGURE V-7

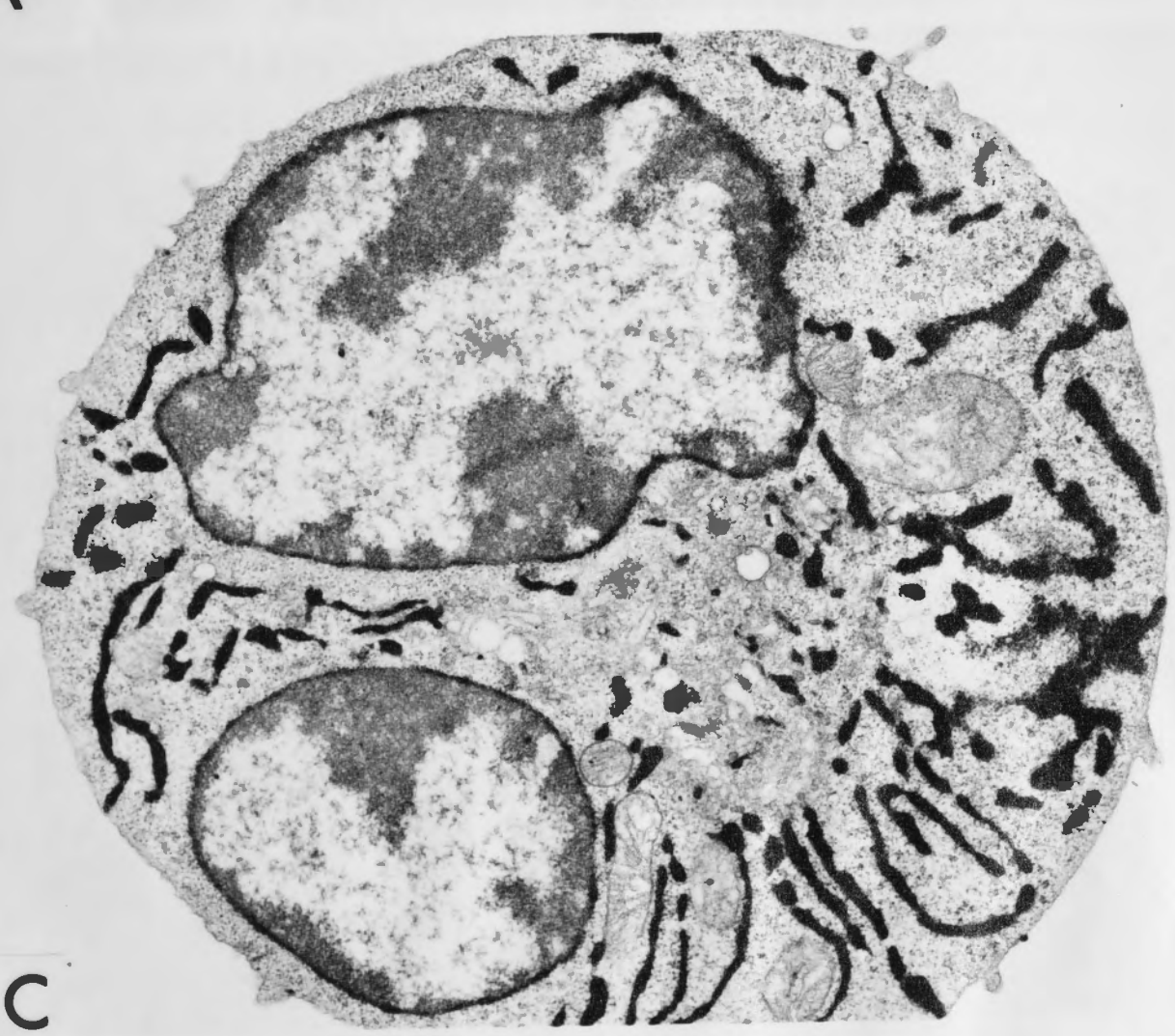
Electron micrographs of lymph cells containing large quantities of antibody to peroxidase.

Magnifications: A X 12,000
B X 10,900
C X 15,500



A

B



C

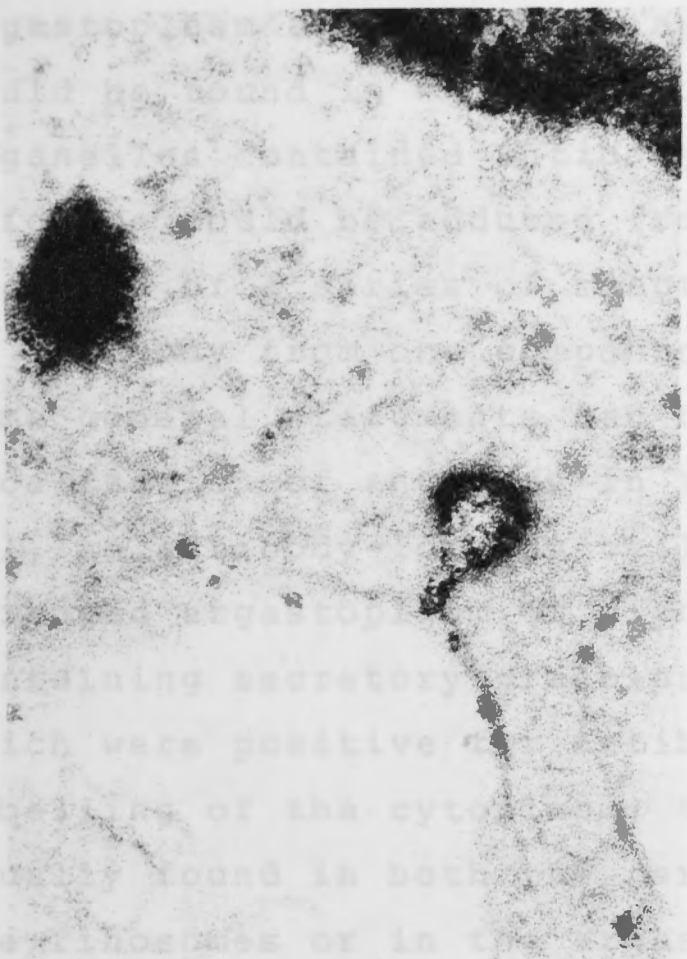
FIGURE V-8

Electron micrographs of lymph cells containing antibody to peroxidase.

A. The reaction product appears to be within the ergastoplasm or a vesicle associated with the plasma membrane. Magnification X 120,000.

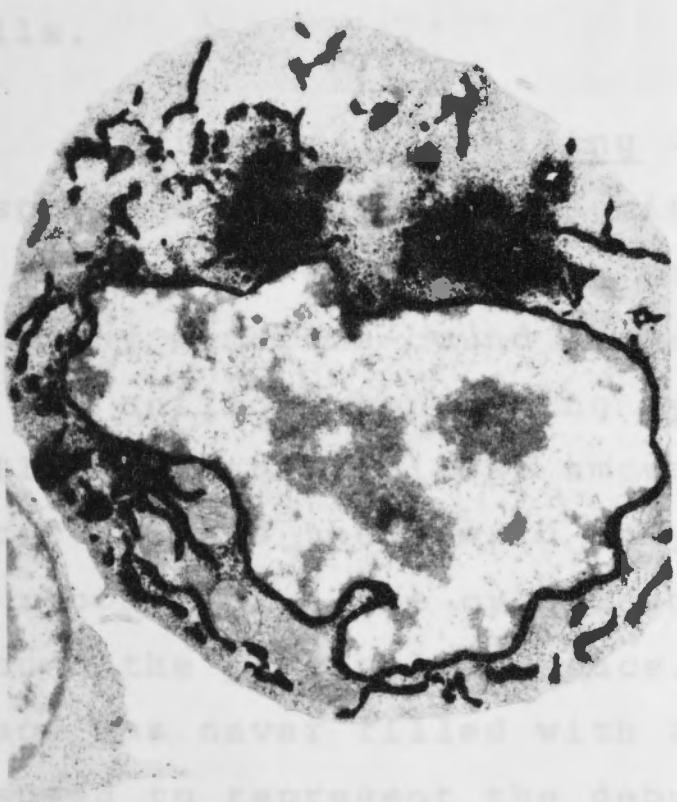
B. The Golgi region is negative for antibody. Magnification X 11,500.

C,D The Golgi region is positive for antibody. Magnification X 9,600.



A

B



C

D

on polyribosomes and in the perinuclear space, the ergastoplasm and the Golgi apparatus. Examples of cells could be found in which one or any combination of these organelles contained antibody. For this reason no convincing evidence could be adduced from the electron microscope pictures of a series of reactions which involved the transfer of antibody from one component of the cell to another. Some general statements can be made however, concerning the localization of antibody in the cells in lymph. First of all, no antibody-forming cells were seen with concentrically arranged ergastoplasm or with dilated ergastoplasmic sacs containing secretory granules; secondly, no blast cells which were positive for antibody showed generalized ribosomal labelling of the cytoplasm; thirdly, while antibody was usually found in both the perinuclear space and either on the ribosomes or in the ergastoplasm, cells could be found with positive cytoplasmic ribosomes and negative perinuclear space and vice versa; fourthly, the Golgi apparatus was negative for antibody in the majority of antibody-containing cells.

Ribosomal labelling . Many blast cells showed small discrete areas of cytoplasmic labelling related to clusters of ribosomes, the reaction product not being sequestered into any membrane-bound structure. The unaggregated ribosomes of the antibody containing lymphocytes were negative. The cells with the smallest amount of antibody that could be detected were those with only a few discrete positive areas of ribosomes in the cytoplasm and some positive focal points around the perinuclear space. In these cells the perinuclear space was never filled with antibody. This localization is assumed to represent the debut of antibody synthesis. In those cells that had antibody in the Golgi apparatus, the ribosomes adjacent to the sacs were usually positive; this may have been due to the diffusion of the reaction products from the Golgi sacs into the surrounding cytoplasm as the antibody appeared to be strongly concentrated there.

Ergastoplasm · Most of the antibody present in the positive cells was contained within the ergastoplasm. The extent to which this structure was filled with antibody varied in the blast cells from a few short, positive profiles to an extensive insinuating network of positive lamellae. In many sections the ergastoplasm appeared to radiate from the centrosomal region towards the peripheral cell membrane, while in other planes, it appeared as numerous short disconnected profiles. The ergastoplasm of the lymphoctyes was usually very poorly developed and was often no more than a few short positive segments. There was no evidence of Russel body formation in any of the cells, and the ergastoplasm was never widely dilated as it was in most plasma cells. Interconnections between the cytoplasmic ergastoplasm and the perinuclear space were seen in some sections and in many of these instances the reaction product was continuous throughout the two regions. In many of the blast cells the profiles of ergastoplasm surrounded mitochondria.

The intensity of the reaction product varied in different parts of the ergastoplasm giving the impression that the concentration of antibody was not uniform within the space enclosed by the rough surfaced reticulum. As well as this, many cells which contained only a small amount of antibody had strongly positive segments of ergastoplasm alongside negative segments. This suggested that, in addition to the concentration of antibody varying within the ergastoplasm antibody synthesis may be occurring in restricted regions along the endoplasmic reticulum. In many sections, segments of ergastoplasm containing antibody ran right to the peripheral cell membrane and in some cases, pictures suggesting excretion of antibody by way of the endoplasmic reticulum were obtained. However, no unequivocal connections were seen between the lumen of the ergastoplasm and the exterior of the cell.

Perinuclear space · The entire perinuclear space was filled with antibody in many of the positive blast cells and in a few it was the only positive structure. In other

cells only small points along the length of the perinuclear space was positive while the rest was negative. In these cells it appeared that antibody synthesis was just beginning and that it was initiated at quite discrete locations around the nucleus. In the lymphocytes the perinuclear space usually contained most of the antibody although even in many of these cells, focal accumulations of antibody occurred around the nucleus and in the cytoplasm.

Golgi apparatus · The Golgi apparatus contained antibody in only a small proportion of the positive cells in lymph. In cells which had a very extensive positive ergastoplasm, there was no evidence that the Golgi was involved necessarily in the transfer of antibody within the cell. In those cells in which it was positive, however, the reaction product in the Golgi sacs was always intense, suggesting that the concentration of antibody was high.

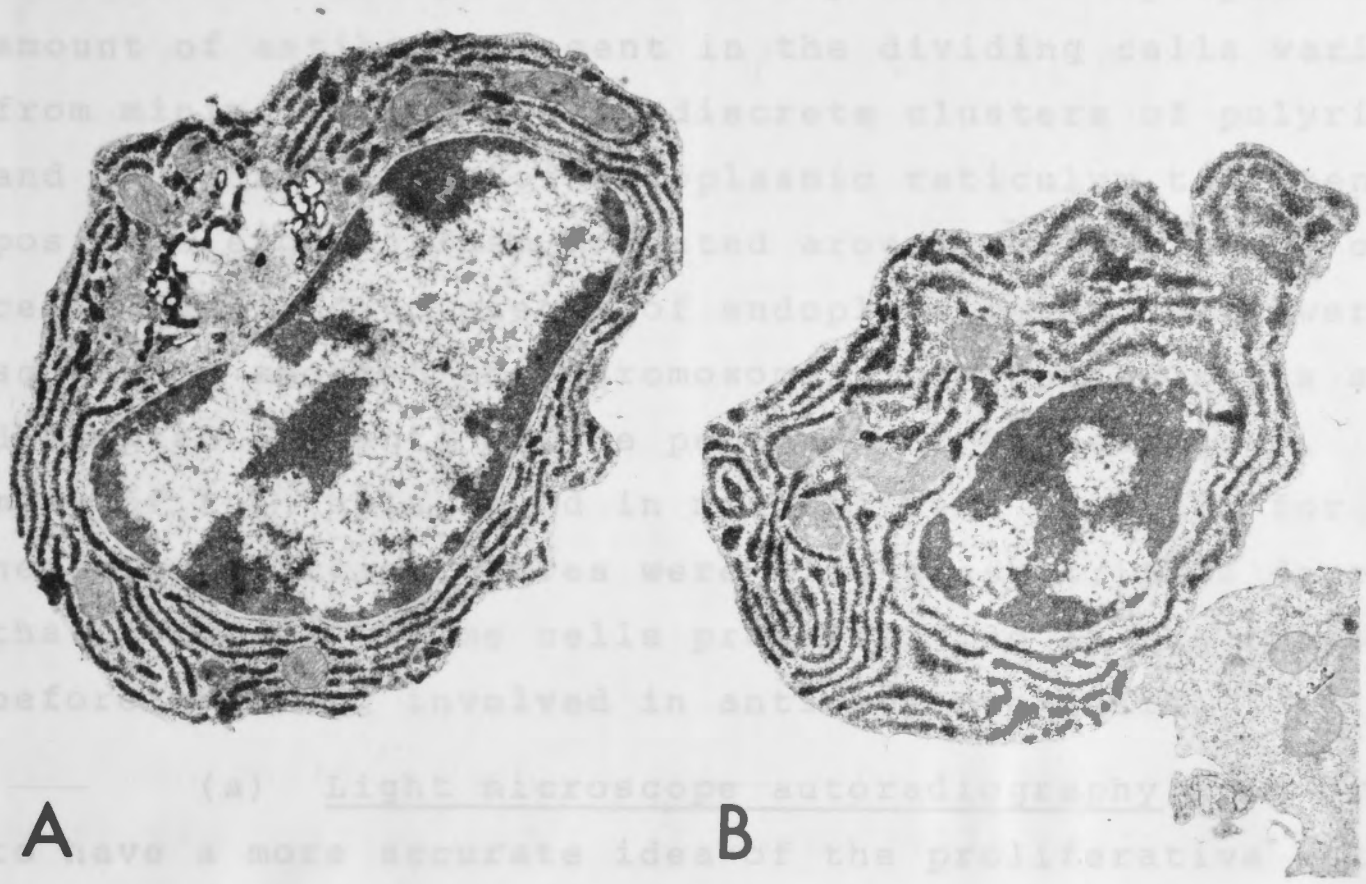
Nuclear labelling · Many of the cells in lymph that contained antibody in their cytoplasm also had a positive nuclear reaction which was confined to the nucleoli. The nucleoli were also found to be labelled in some cells that had no antibody in the perinuclear space or in the ergastoplasm. Nucleolar labelling was not seen in the control specimens or in the positive plasma cells found in lymph nodes. The reaction product was difficult to localize precisely in the nucleoli although in some cases it appeared to be associated with ribosomal particles.

(b) Cells in Lymph nodes. In lymph nodes removed after the immune response had died away in the lymph, all the cells which contained antibody fell within the category of plasma cells (Figure V-9). The antibody was located mostly in concentric lamellated ergastoplasmic sacs, some of which were dilated to give the appearance of Russel bodies. The perinuclear space in the majority of these cells was negative as was frequently the Golgi apparatus. Some mature plasma cells, negative for anti-peroxidase antibody were also present in the lymph nodes.

FIGURE V-9

Electron micrographs of plasma cells containing antibody to peroxidase obtained from a suspension of lymph node cells.

Magnifications:
A X 9,100
B X 10,400
C X 10,400
D X 10,400


A
B
C
D

The proliferation of antibody-forming cells

Throughout the response cells in mitosis could be identified in both light microscope smears and in the electron microscope with antibody in their cytoplasm. The amount of antibody present in the dividing cells varied from minimal labelling in discrete clusters of polyribosomes and in short pieces of endoplasmic reticulum to extensive positive ergastoplasm oriented around the periphery of the cell. Rounded up pieces of endoplasmic reticulum were often scattered amongst the chromosomes in prophase cells suggesting disrupted segments of the perinuclear ergastoplasm. Although many of the cells found in mitosis were positive for antibody, negative mitotic figures were also present; this demonstrated that there were some cells proliferating in the response before becoming involved in antibody synthesis.

(a) Light microscope autoradiography. In order to have a more accurate idea of the proliferative potential of the cells in lymph and to establish the number of cells which entered the S phase of the mitotic cycle at the same time as they were synthesizing antibody, samples of lymph were incubated with ^{3}H-thymidine and the antibody-forming cells revealed with diaminobenzidine and hydrogen peroxide.

After 1 hr incubation with ^{3}H-thymidine between 70 - 80 per cent of the large blast cells were found to have incorporated the radioactive label into their nuclear DNA. At the peak of the response when the maximum number of antibody-forming cells was present, it was found that the majority of the blast cells were both incorporating thymidine and synthesizing anti-peroxidase antibody. As a group, the smaller antibody-positive cells did not incorporate thymidine to the same extent as the larger blast cells. In some of these blast cells the reaction product was very light suggesting that antibody synthesis had been proceeding for only a short time. On the other hand there was a good number of cells staining intensely for anti-peroxidase antibody which were also synthesizing DNA. This demonstrated that active antibody synthesis and the presence of a high content

of antibody in the cell was not incompatible with the division of the cell.

(b) Electron microscope autoradiography. It was apparent from the ^{3}H-thymidine studies that, for the majority of the blast cells that appeared in lymph during the secondary response to horse-radish peroxidase, antibody synthesis and cell division went hand in hand. These cells can therefore be considered as differentiated cells in the biochemical sense but undifferentiated cells in the ultrastructural sense. The plasma cells in the lymph node on the other hand were highly differentiated cells in both contexts. To test whether the development of a highly organized ergastoplasm storing specific antibody limited the capacity of a cell to synthesize DNA, cells were collected coming from popliteal nodes of sheep hyper-immunized with Salmonella. With this antigen, as with influenza virus (Smith and Morris, 1970), it is possible to find a proportion of antibody-forming cells in the lymph at the peak of the response with the ultrastructure of mature plasma cells.

Samples of lymph were obtained during vigorous secondary responses to Salmonella, the cells were incubated with ^{3}H-thymidine and then exposed for light and electron microscope autoradiography. Examples of cells in all stages of ultrastructural differentiation could be found synthesizing DNA (Figures V-10, V-11). In addition however to the abundant blast cells, cells that could only be classified as mature plasma cells on cytological and immunological grounds were also found to incorporate ^{3}H-thymidine as actively as many of the immature blast cells. Such mature plasma cells included those with dilated ergastoplasm filled with secretion products as well as cells with Russel bodies.

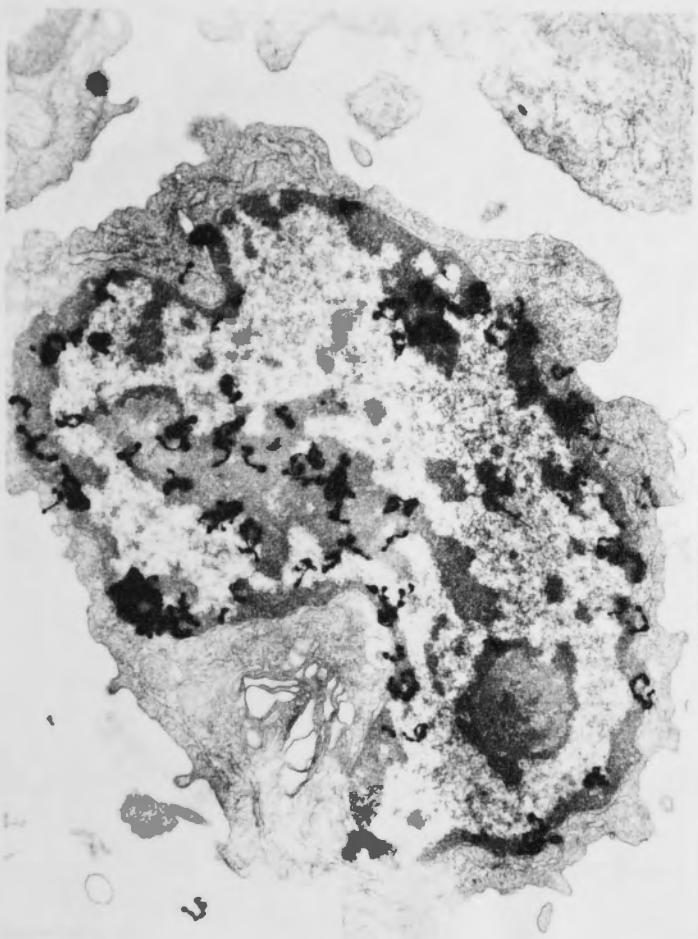
Discussion

The term "undifferentiated cell" implies that a cell, given the appropriate stimulus has the potential to develop in several separate directions. Is the lymphocyte then,

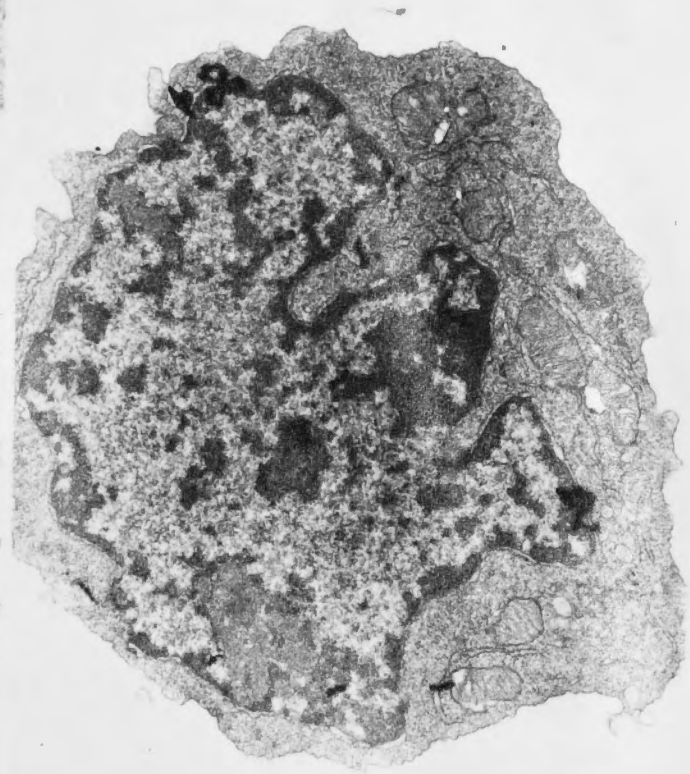
FIGURE V-10

Electron microscope autoradiographs of lymph cells collected during an immune response to Salmonella showing the appearance of cells incorporating ^{3}H-thymidine.

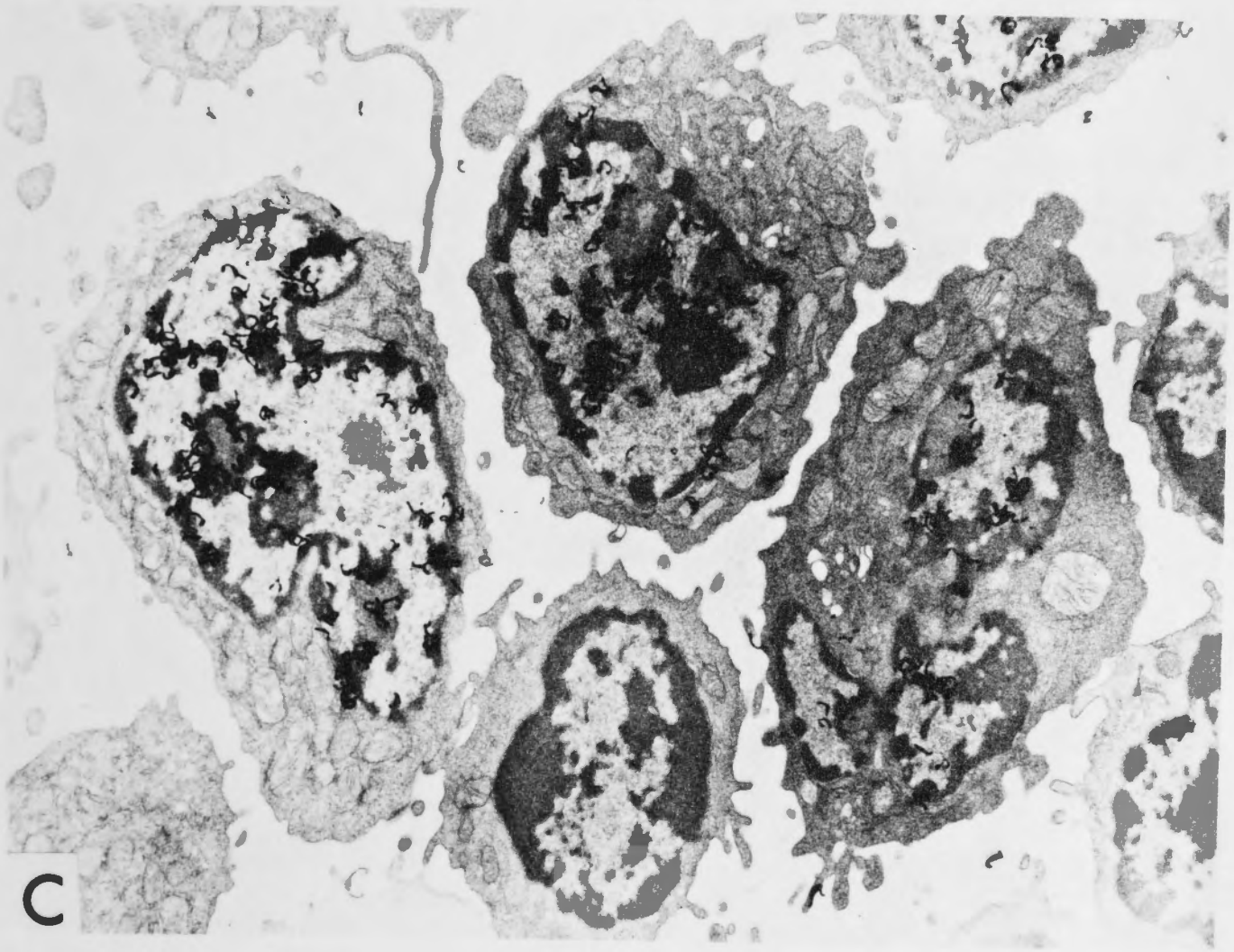
Magnifications: A X 11,800
B X 11,800
C X 9,200



A



B

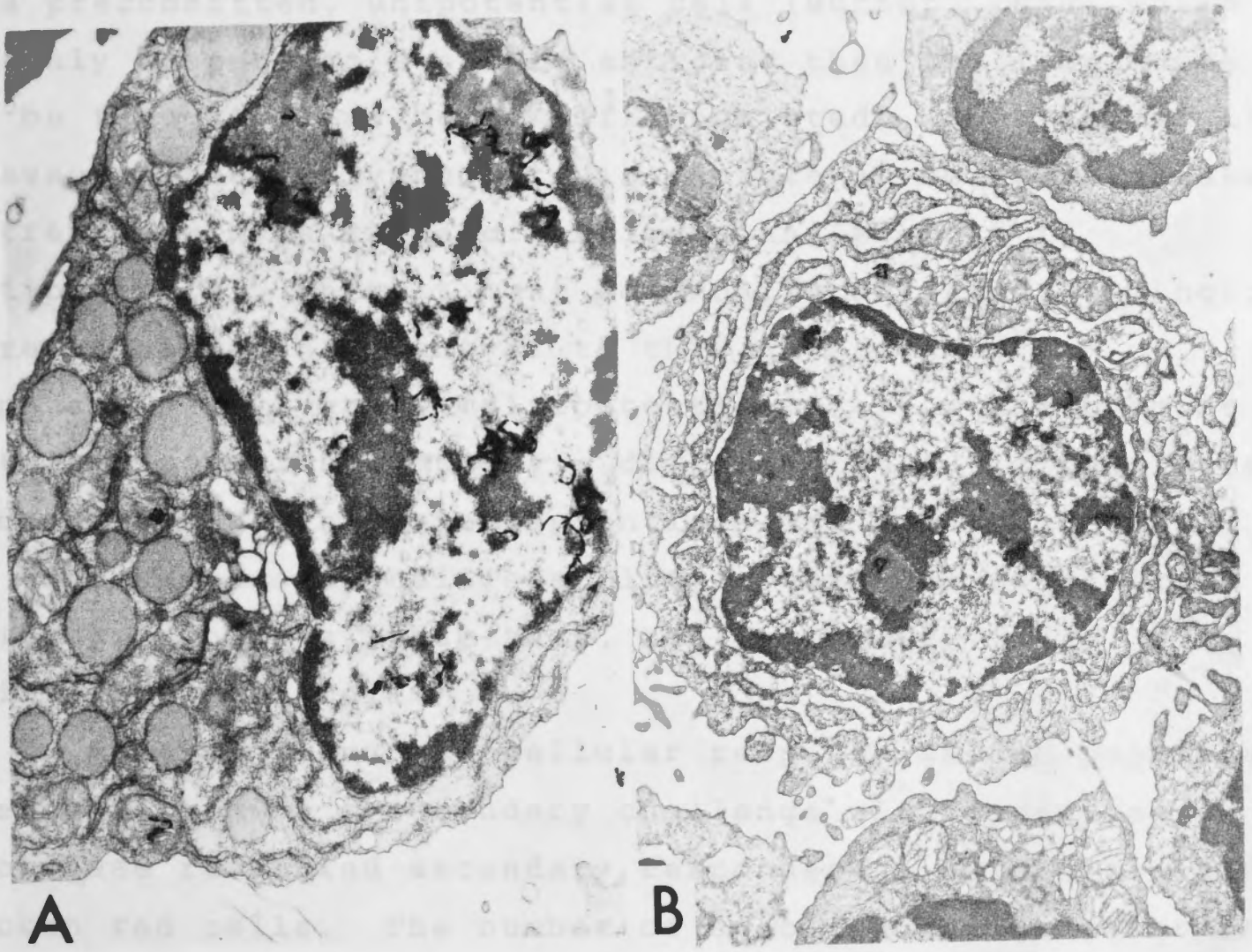


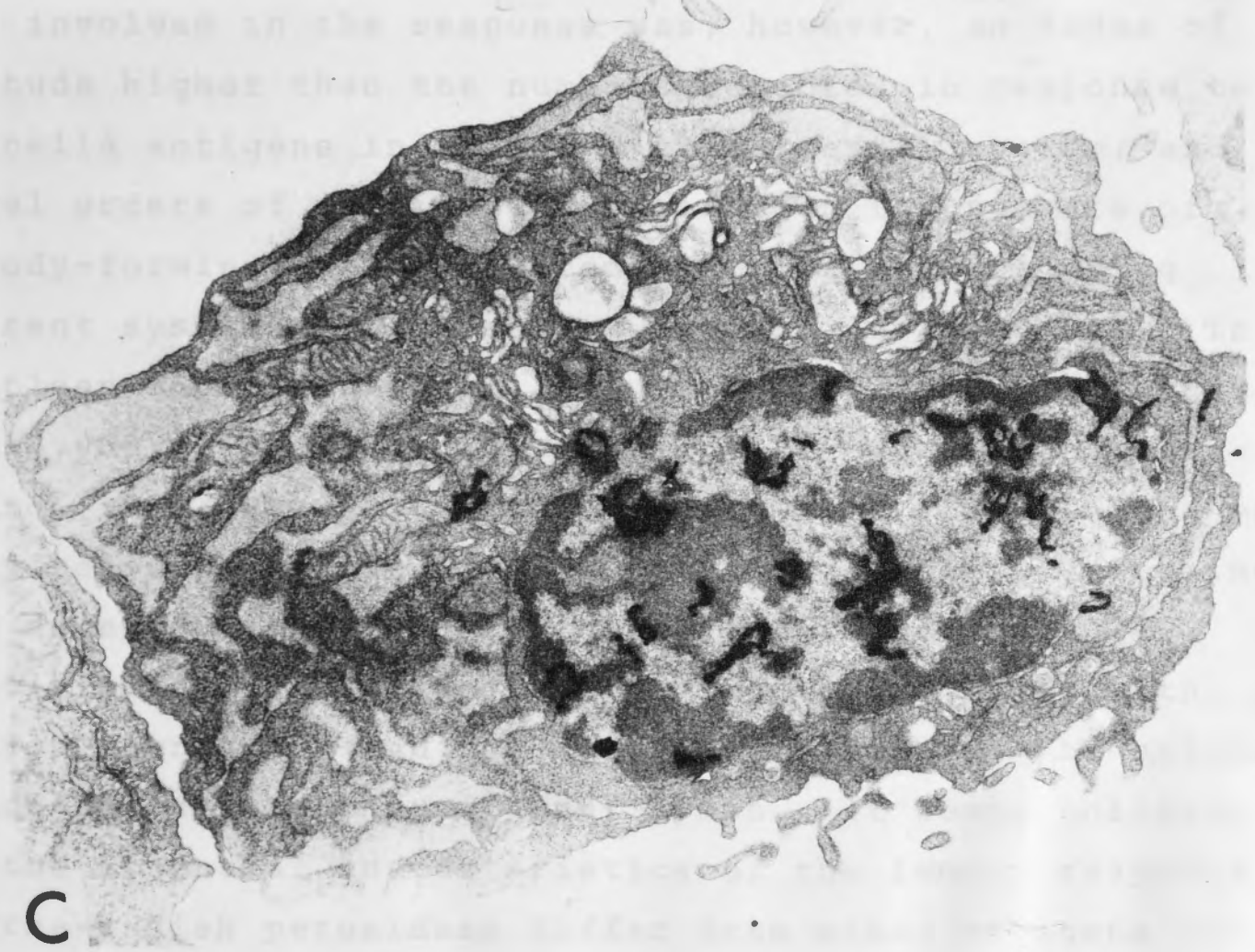
C

FIGURE V-11

Electron microscope autoradiographs of plasma cells obtained from lymph during an immune response to Salmonella.

Magnifications: A X 12,600
B X 13,700
C X 18,200


A
B


C

a differentiated cell? If it is assumed that a lymphocyte is a precommitted, unipotential cell (Burnet, 1959) which can only respond to a single antigen, then the lymphocyte can be thought of as being differentiated. However, if it is assumed that a lymphocyte is capable of being stimulated to transform by a range of antigens and that it is multipotential, then it must be undifferentiated. Although there is no definite answer to this question of unipotentiality versus multipotentiality, for the purpose of this discussion lymphocyte differentiation is considered to be a process initiated by antigen and concerned with the expression of a cell's ability to synthesize specific antibody. In this sense then, the lymphocyte is an undifferentiated cell.

In general terms the cellular response in the popliteal efferent lymph to a secondary challenge with horse-radish peroxidase resembled secondary responses to Salmonella and chicken red cells. The number of specific antibody-forming cells involved in the response was, however, an order of magnitude higher than the numbers detected in response to Salmonella antigens in the same experimental system, and several orders of magnitude greater than the numbers of antibody-forming cells reported by other workers using different systems. Figures for antibody-forming cells in the spleen and lymph nodes are usually of the order of 1:1,000 to 1:10,000 (Jerne, Nordin and Henry, 1963; Ingraham and Bussard, 1964; Harris, Hummeler and Harris, 1966). The most likely reasons for the high proportion of antibody-forming cells detected in the present experiments were the sensitivity of the immunochemical reaction coupled with electron microscopy and the specific release of the antibody-forming cells into the efferent lymph. It seems unlikely that the essential characteristics of the immune response to horse-radish peroxidase differ from other antigens and the proportion of antibody-forming cells to the total number of differentiating cells produced in secondary immune responses to other antigens may well be as high or higher than the figures found here for responses to horse-radish peroxidase

provided sufficiently sensitive systems were available for their detection. Any explanations of the cellular basis for antibody production will need to recognise that at the height of an immune response, more than 40 million antibody-forming cells may leave the popliteal lymph node each hour via the lymph and these cells may represent about 20 per cent of the total cells and about 75 per cent of the transforming cell population.

The exact relationship between antibody-forming cells and other types of antigen-sensitive cells and antibody-forming cell precursors is uncertain but current immunological thought appears to favour the idea that an interaction occurs between antigen and distinct populations of lymphoid cells from the thymus and bone marrow which, in some way, leads to the development of differentiated plasma cells which make antibody (Claman, Chaperon and Triplett 1966a, 1966b; Davies, Leuchars, Wallis and Koller, 1966; Davies, Leuchars, Wallis, Marchant and Elliott, 1967; Miller and Mitchell, 1968; Mitchell and Miller, 1968a; Nossal, Cunningham, Mitchell and Miller, 1968). There is good evidence that the antibody-forming cells are the outcome of cellular proliferation (Baney, Vazquez and Dixon, 1962; Tannenberg, 1967; Claflin and Smithies, 1967; Szenberg and Cunningham, 1968; Tannenberg and Malaviya, 1968) although it is held that the most extensive proliferation occurs amongst "antigen-sensitive" cells and "antibody-forming cell precursors" (Mäkelä and Nossal, 1962; Nossal and Mäkelä, 1962; Metcalf, 1967). The actual antibody-forming cells were thought to have only limited proliferative potential once antibody synthesis had begun (Nossal, 1962). Both "antigen-sensitive cells" and "antibody-forming cell precursors" have been shown to be present in lymph as part of the recirculating cell population (Mitchell and Miller, 1968a). The present results show two things clearly. The first is that most of the cells in lymph that show evidence of transformation in response to antigen are proliferating cells and the second is that most of these cells are also actually synthesizing antibody. In view of the joint commitment of most of the transforming cells in

lymph to division and antibody synthesis it is difficult to find a place for "antigen-sensitive cells" and "antibody-forming cell precursors" among the proliferating cell population in lymph.

The immune response to horse-radish peroxidase has been studied extensively in the spleen and the popliteal lymph nodes of rabbits by Leduc, Avrameas and Bouteille (1968); Leduc, Scott and Avrameas (1969); and Avrameas and Leduc (1970). Their observations on the cellular basis of antiperoxidase antibody synthesis is of relevance to the present experiments. They concluded initially that the cells in lymph nodes and spleen involved in antibody synthesis were for the most part cells of the plasma cell series; haemocytoblasts, plasmablasts, immature and mature plasma cells. In all these cells, antibody was localized specifically in the perinuclear space, the endoplasmic reticulum, and the Golgi apparatus. Leduc, Scott and Avrameas (1969) identified another type of antibody-forming cell in paraformaldehyde fixed tissue which they termed the lymphoblast. This type of cell showed uniform cytoplasmic staining involving all the ribosomes and no localization of antibody in the endoplasmic reticulum. Subsequently they renamed this cell a large lymphocyte (Avrameas and Leduc, 1970). Avrameas and Leduc (1970) also proposed another type of antibody-forming cell which they described as a specialized lymphocyte and to which they gave the name "lymphoplasmacyte". This type of cell was described as having the elaborate ergastoplasm of the plasma cell but the dense cytoplasm and the hyperchromatic nucleus of the small lymphocyte. They make no mention of identifying lymphocytes as antibody-forming cells, although this cell has been implicated in antibody synthesis by several groups of workers (Attardi, Cohn, Horibata and Lennox, 1959; Attardi, Cohn, Horibata and Lennox, 1964; Cunningham, Smith and Mercer, 1966; Gowans and McGregor, 1965). Irrespective of what terminology is given to the cells synthesizing antibody, it is certain that the active cell population in lymph is very different from its counterpart in the node.

Hummeler, Harris, Tomassini, Hechtel and Farber (1966) and Cunningham et al (1966) compared antibody-forming cells in the lymph and in lymph nodes using the haemolytic plaque assay to isolate the active cells. Because of technical problems associated with manipulation of single cells the number of antibody-forming cells that they examined was small. Hummeler et al (1966) differentiated antibody-forming cells in the lymph and blood from those in lymph nodes on the basis of their size and morphology and concluded that the active cells in lymph and blood were small and were lymphocytes, while those in the nodes were large cells and were plasmacytes. Their findings for the antibody-forming cells in lymph are therefore quite different from the present results as the majority of antibody-forming cells in lymph are large blast cells, the lymphocytes being only a minor component of the active population. The cells Cunningham et al (1966) identified in the lymph by plaque assay were similar to those found in the present experiments and are identical with those described by Hall and Morris (1963); Hall, Morris, Moreno and Bessis (1967); and Smith and Morris (1970) in immune responses to a wide variety of antigens.

The localization of antibody within antibody-forming cells.

Antibody appeared to be discretely and predictably located within several different ultrastructural organelles and this was characteristic for all types of antibody-forming cells. In those cells which contained a minimum amount of antibody the localization was confined to clusters of ribosomes and in restricted areas of the perinuclear space and endoplasmic reticulum but never generalized throughout the cytoplasm. Avrameas and Bouteille (1968); Leduc, Avrameas and Bouteille (1968) and Avrameas and Leduc (1970) deduced from histological evidence that antibody synthesis begins immediately around the nucleus and that antibody protein accumulates in the perinuclear space and subsequently fills the ergastoplasm. They make no comment on the sites at which antibody synthesis first occurs in those cells showing

uniform ribosomal staining but the initiation of specific protein synthesis in these cells must presumably be a different phenomenon. They claimed that antibody synthesis occurs in waves, the antibody travelling outwards from the nucleus towards the periphery of the cell by way of the endoplasmic reticulum and Golgi, where it is excreted by the process of clasmacytosis (Thiery, 1960).

It is not possible in the present type of experiment to define the sequence of synthesis and transport of antibody through the ultrastructural components of the cells. There is no evidence available for the rate of turnover of antibody proteins in any of the organelles and while it is possible that the sequence of events in plasma cells is similar to that described for other protein synthesizing cells such as in the exocrine pancreas (Siekevitz and Palade, 1958), the situation may in fact be quite different. It is possible however to make certain specific statements concerning antibody synthesis in free-floating lymph cells. If antibody synthesis is initiated in the perinuclear endoplasmic reticulum, synthesis can extend to the cytoplasm at a very early stage, long before the perinuclear space contains much antibody. The Golgi apparatus of the blast cells was positive for antibody in only a very small proportion of the cells in contradistinction to the plasma cells in the node (Leduc, Scott and Avrameas, 1969). If we assume that the antiperoxidase antibody is being excreted by the blast cells, as antibody against Salmonella and red cell antigens is excreted by them, then we can say that the Golgi apparatus is not necessarily involved in antibody excretion in these cells even though it does appear to play a role in concentrating antibody in some cells.

The nucleoli of many cells synthesizing antibody in the lymph developed a positive reaction for peroxidase. On occasions both blast cells and lymphocytes with no detectable antibody in their cytoplasm also gave a positive reaction in their nucleoli. The nucleoli of the mature plasma cells in the lymph nodes were not found to be positive. The significance of this phenomenon is not known but it may

represent the earliest symptom of antibody synthesis by an activated cell.

Immunological memory

The possibility exists that any of the cells which appear as antibody-forming cells may become circulating memory cells. For this to occur these cells would have to revert to the appearance of lymphocytes as these are essentially the only cells circulating in lymph. The studies with ^{3}H-thymidine showed that the processes of cell differentiation and antibody synthesis occur hand in hand and that at all levels of ultrastructural organization, DNA synthesis is taking place, even in what might be thought terminally differentiated plasma cells. This illustrates that for the cells in lymph, at any rate, biochemical and ultrastructural differentiation does not inhibit DNA synthesis and that the cells in this environment cannot be considered end cells. The characteristics of the progeny of these differentiated cells is not known, but there is no reason to assume that cell division in any of the spectrum of transforming cells would give rise necessarily to an ultrastructurally more differentiated daughter cell, particularly as the environment in which they find themselves may now be quite different from that which held in the lymph node after the antigenic challenge.

The development of the mature plasma cell can be considered as a series of sequential differentiations which occur particularly in the medulla of the lymph nodes where antigen is localized. No information has been forthcoming on the effects of continuing antigenic stimulation on the differentiation of cells of the lymphoid or plasma cell series but it seems certain that in a challenge with antigen and incomplete Freund's adjuvant, the cells within the node will be subjected to antigenic stimulation for a longer time than the cells which escape into the lymph. Additionally and perhaps more importantly, the plasma cells which remain fixed in the node have an opportunity to modify their environment and alter the processes of cell differentiation

by their own metabolic activities. Antibody excreted by the plasma cells into the surrounding tissue fluid may react with new antigen to give antigen-antibody complexes, or antibody itself may build up in concentrations in the immediate vicinity of the cells and affect further excretion of the antibody. Whatever the events in the node, it is certain that the free-floating cells exist in a different environment from the antibody-forming cells of the node and while in the lymph they cannot modify this environment. In addition should they localize at a later date in another area of fixed lymphoid tissue, it is unlikely that the conditions there will duplicate those in the node from which they originated. Thus in terms of these general considerations, regardless of the progenitors of the antibody-forming cells in the lymph and in the node, their development will proceed under different modifying stimuli. The antibody-forming cells of lymph need not be committed irrevocably to terminal plasma cell differentiation and in view of the minor ultrastructural changes that occur in many of the cells up to the time the first antibody is synthesized in them, withdrawal from this activity would require only minimal ultrastructural changes to effect a reversion to the small lymphocyte which could then act as a circulating memory cell.

Summary

1. Comparison of the properties of cell populations leaving a stimulated lymph node at various stages of the immune response confirmed that the characteristics of the population changed during the response. Antigen-stimulated cells early in the response included a higher proportion of cells that had the appearance of small lymphocytes, contained only small amounts of antibody but actively incorporated ^{3}H-thymidine. Cells later in the response were primarily large, blast cells which contained more antibody, and also actively incorporated ^{3}H-thymidine.

2. The use of horse-radish peroxidase as an antigen resulted in immune responses in which as many as 70-80 per cent of the blast cells that appeared in the efferent popliteal lymph contained specific antibody at the peak of the response. With the aid of the electron microscope the localization of antibody was observed to be associated within a variety of cytoplasmic structures within cells. A positive reaction for antibody also occurred in the nucleoli of some cells. After the response in the lymph had died away cells containing antibody could be found in the lymph node, these had the characteristics of plasma cells and were quite different from the antibody-containing cells in the lymph.

3. The majority of the blast cells that contained antibody also incorporated ^{3}H-thymidine; many cells resembling mature plasma cells ultrastructurally, also incorporated ^{3}H-thymidine into their nuclear DNA.

4. It seemed likely that the majority of cells that appeared in the lymph after an antigenic challenge were involved in antibody synthesis at some stage of their life history.

CHAPTER VI

THE EFFECT OF HETEROLOGOUS ANTI-LYMPHOCYTE SERUM ON ANTIGEN-STIMULATED CELLS

The Effect of Heterologous Anti-Lymphocyte Serum on Antigen-Stimulated Cells

The immunosuppressive activity of heterologous anti-lymphocyte serum (ALS) has been well documented both experimentally and clinically (see review by Medawar, 1969). It is not known precisely how ALS exerts its effect or what is the relationship between the lymphopoenia which it induces and the immunosuppressive effect.

The present experiments were designed primarily to investigate the effects of an ALS on the production of the antigen-stimulated cells which appear in the efferent lymph during an immune response. It was thought that if antigen-stimulated cells were eliminated by the local administration of specific antiserum it might be possible to abort the immune response. The initial experiments were carried out to determine some of the properties of anti-lymphocyte serum and to study its effect on the unstimulated popliteal lymph node and on the cells leaving it.

Results

The Effects of ALS on Normal Lymphocytes Treated in Vitro

Agglutinating and Cytotoxic Activity

EXPERIMENTAL

Normal lymphocytes were obtained from the efferent popliteal lymph of a sheep and were incubated at 37°C in normal rabbit serum, rabbit ALS, anti-lymphocyte globulin (ALG), or rabbit antiserum directed against sheep serum proteins. Incubations were done in the absence of complement to assay for cell agglutination and in the presence of complement to assay for cytotoxicity by the dye exclusion method. In addition, the capacity of cells to give a normal lymphocyte transfer (NLT) reaction after exposure to ALS, was tested by incubating lymphocytes *in vitro* for 30 minutes at 37°C with either normal rabbit serum or with a 1:2 or a 1:100 dilution of ALS. The cells were injected

into the skin of a recipient sheep in a dose of 10 million cells per injection site. Each sample of cells was injected in three separate sites.

RESULTS

The potency of the anti-lymphocyte sera produced in different rabbits varied considerably even though the same procedure for immunization was used in all cases. All the samples of heterologous ALS were capable of agglutinating or causing the lysis of sheep lymphocytes (Table VI-1) and approximately 50 per cent of the cells were dead after 30 minutes and virtually all the cells dead after 60 minutes exposure.

Figure VI-1 shows the capacity of ALS treated cells to produce an NLT reaction. Exposure to a 1:2 dilution of ALS destroyed the capacity of the cells to produce a lesion and exposure to a 1:100 dilution also reduced the reaction. This test was more sensitive than the dye exclusion method because the dilution of 1:100 ALS did not result in any detectable increase in the number of cells which took up the dye.

Lymphocyte Transformation

In the absence of complement, ALS agglutinated lymphocytes but did not destroy them. It has been shown by Gräsbeck, Nordman and de la Chapelle (1964) and others that when lymphocytes are cultured in the presence of ALS under optimal growth conditions *in vitro*, they undergo transformation. This phenomenon was examined using sheep lymphocytes.

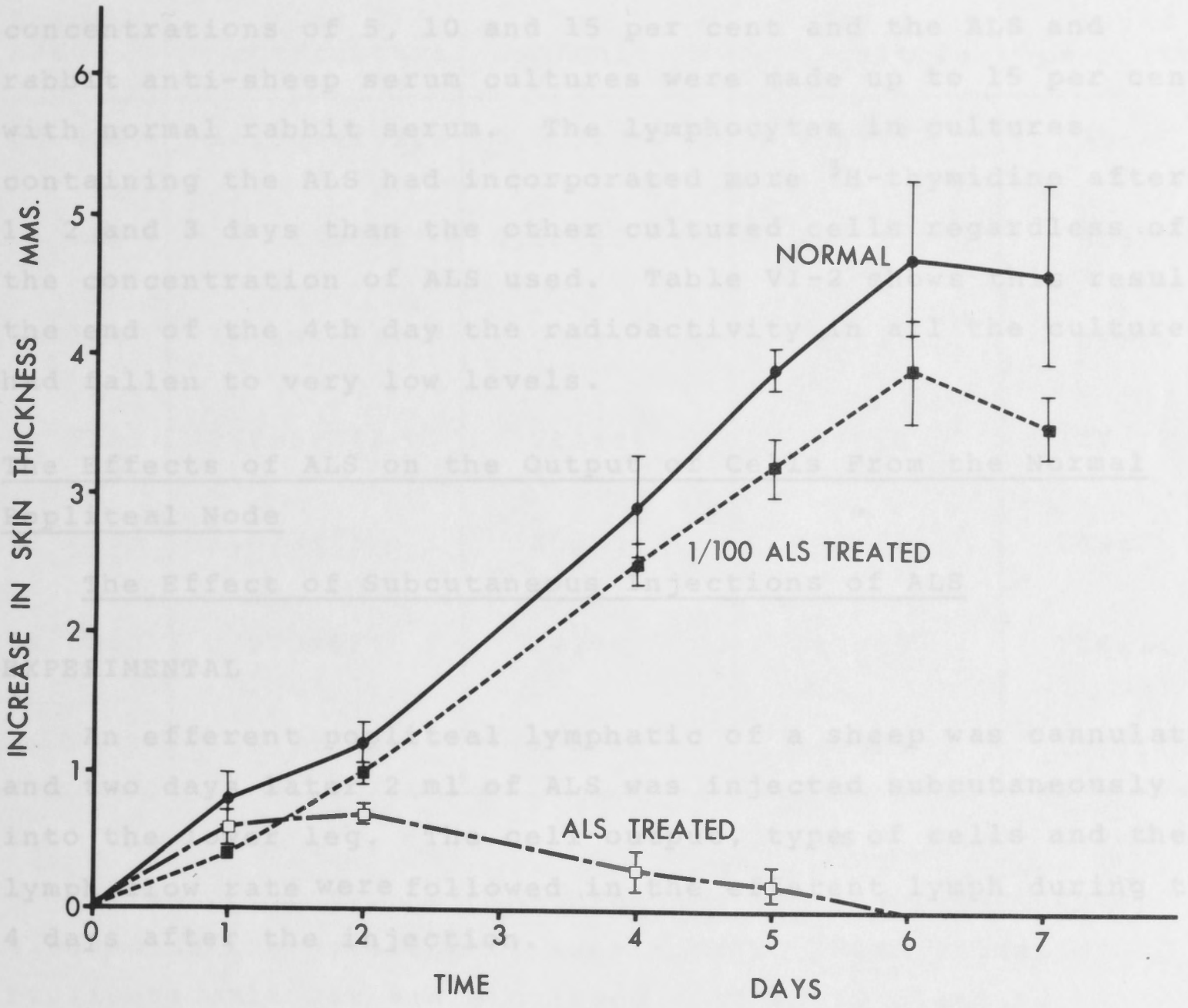
Cultures of normal efferent lymph cells were set up in various concentrations of ALS to determine the concentration at which the maximum degree of transformation occurred. Cultures were done in duplicate and ALS concentrations of 0, 1.25, 2.5, 5, 10, 15, 20 and 40 per cent were tested. The final concentration in all cultures was adjusted to 15 per cent by the addition of normal rabbit serum except in those cultures in which 20 per cent and 40 per cent ALS was used. ^{3}H-thymidine (3μCi/culture) was added 16 hours before the end of the 3 day culture period. It was found

TABLE VI-1

	Number of Lymphocytes Viable after 30 min (%)	Lymphocyte Agglutination Titre (Reciprocal)
Normal rabbit serum	95	0
Rabbit anti-sheep serum	91	0
Rabbit anti-lymphocyte serum (pooled)	51	640
(individual range)	-	20 - 1280
Rabbit anti-lymphocyte serum (pooled gamma-globulin fraction)	53	320

A comparison of the cytotoxic and agglutinating activity of rabbit sera on sheep lymphocytes.

FIGURE VI - 1



The effects of treatment with ALS and complement on the ability of homologous lymphocytes to produce an NLT reaction.

that the maximum degree of transformation occurred in the cultures containing 10 per cent ALS.

Another experiment was done comparing the effect of ALS, normal rabbit serum and rabbit anti-sheep serum on normal sheep lymphocytes. The sera were tested at concentrations of 5, 10 and 15 per cent and the ALS and rabbit anti-sheep serum cultures were made up to 15 per cent with normal rabbit serum. The lymphocytes in cultures containing the ALS had incorporated more ^{3}H-thymidine after 1, 2 and 3 days than the other cultured cells regardless of the concentration of ALS used. Table VI-2 shows this result. By the end of the 4th day the radioactivity in all the cultures had fallen to very low levels.

The Effects of ALS on the Output of Cells From the Normal Popliteal Node

The Effect of Subcutaneous Injections of ALS

EXPERIMENTAL

An efferent popliteal lymphatic of a sheep was cannulated and two days later 2 ml of ALS was injected subcutaneously into the lower leg. The cell output, types of cells and the lymph flow rate were followed in the efferent lymph during the 4 days after the injection.

RESULTS

Table VI-3 shows the results of this experiment. During the first hour the lymphocyte output of the node fell significantly and over the period from 2-9 hours following the injection, the output of lymphocytes was less than 1 per cent of the preinjection level. The rate of lymph flow increased significantly and at the same time neutrophils and eosinophils appeared in the lymph. The extent of this inflammatory reaction varied with individual sheep being very pronounced in some animals while in others it was hardly detectable. The lymphocyte output had usually returned to resting levels by 10-20 hours after the injections; subsequently the output rose and in most cases surpassed the preinjection level (Table VI-3).

TABLE VI-2

Serum Added	Day 1 cpm/culture	Day 2 cpm/culture	Day 3 cpm/culture	Day 4 cpm/culture
5% ALS	16,069±1,209	5,587±302	7,385±417	155±83
10% ALS	18,414±811	6,681±1,593	6,444±1,080	60±27
15% ALS	15,599±582	4,538±1,594	6,299±681	189±60
5% RASS	2,825±243	211±25	371±10	142±7
10% RASS	1,623±85	203±31	402±36	148±6
15% RASS	970±61	142±9	312±59	176±11
15% NRS	3,447±880	276±20	240±23	127±5

The incorporation of ^{3}H-thymidine by sheep lymphocytes cultured in the presence of rabbit sera. Mean values of triplicate cultures are expressed with their standard deviation.

ALS- anti-lymphocyte serum
RASS- rabbit anti-sheep serum
NRS- normal rabbit serum

TABLE VI-3

			Differential White Cell Counts					
Time hrs.	Flow Rate ml/hr	Total White Cell Output /hr	Lymphocytes %	Blast Cells %	Macrophages %	Neutrophils %	Eosinophils %	Lymphocyte Output %
Before ALS	5.1	6.12×10^7	98.5	1.5	0	0	0	100
0-1	6.6	6.93×10^6	98.0	2.0	0	0	0	11.1
1-2	11.0	1.21×10^6	61.0	0.2	0	32.1	6.7	1.2
2-3	13.2	7.93×10^5	23.2	0	2.9	68.5	5.4	0.3
4-6	14.6	6.39×10^5	41.6	1.4	2.1	50.6	4.3	0.4
6-9	16.7	9.55×10^5	51.0	1.5	3.3	42.2	2.0	0.8
9-21.5	10.2	1.57×10^7	-	-	-	-	-	-
21.5-23.5	5.9	2.61×10^7	81.2	1.6	0.3	16.6	0.3	34.6
23.5-27.5	5.4	6.05×10^7	-	-	-	-	-	-
27.5-29.5	6.1	1.21×10^8	-	-	-	-	-	-
29.5-51.5	5.0	1.64×10^8	96.7	2.4	0	0.5	0.4	259.8
51.5-71.5	4.4	1.43×10^8	-	-	-	-	-	-
71.5-72.5	4.2	1.60×10^8	95.1	4.9	0	0	0	248.4
72.5-82.5	3.7	1.02×10^8	-	-	-	-	-	-
82.5-94	3.5	7.30×10^7	-	-	-	-	-	-
95-98	3.3	1.01×10^8	97.0	3.0	0	0	0	160.1

The effect of a 2 ml subcutaneous injection of ALS on the characteristics of efferent popliteal lymph.

The Effect of Continuous Infusion of ALS and Normal Rabbit Serum

EXPERIMENTAL

To avoid giving a series of subcutaneous injections, experiments were carried out in which ALS or normal rabbit serum was infused directly into popliteal lymph nodes via the afferent lymphatics. These infusions could be continued for 3-4 days and in this way a steady state concentration of ALS could be maintained in the lymph node.

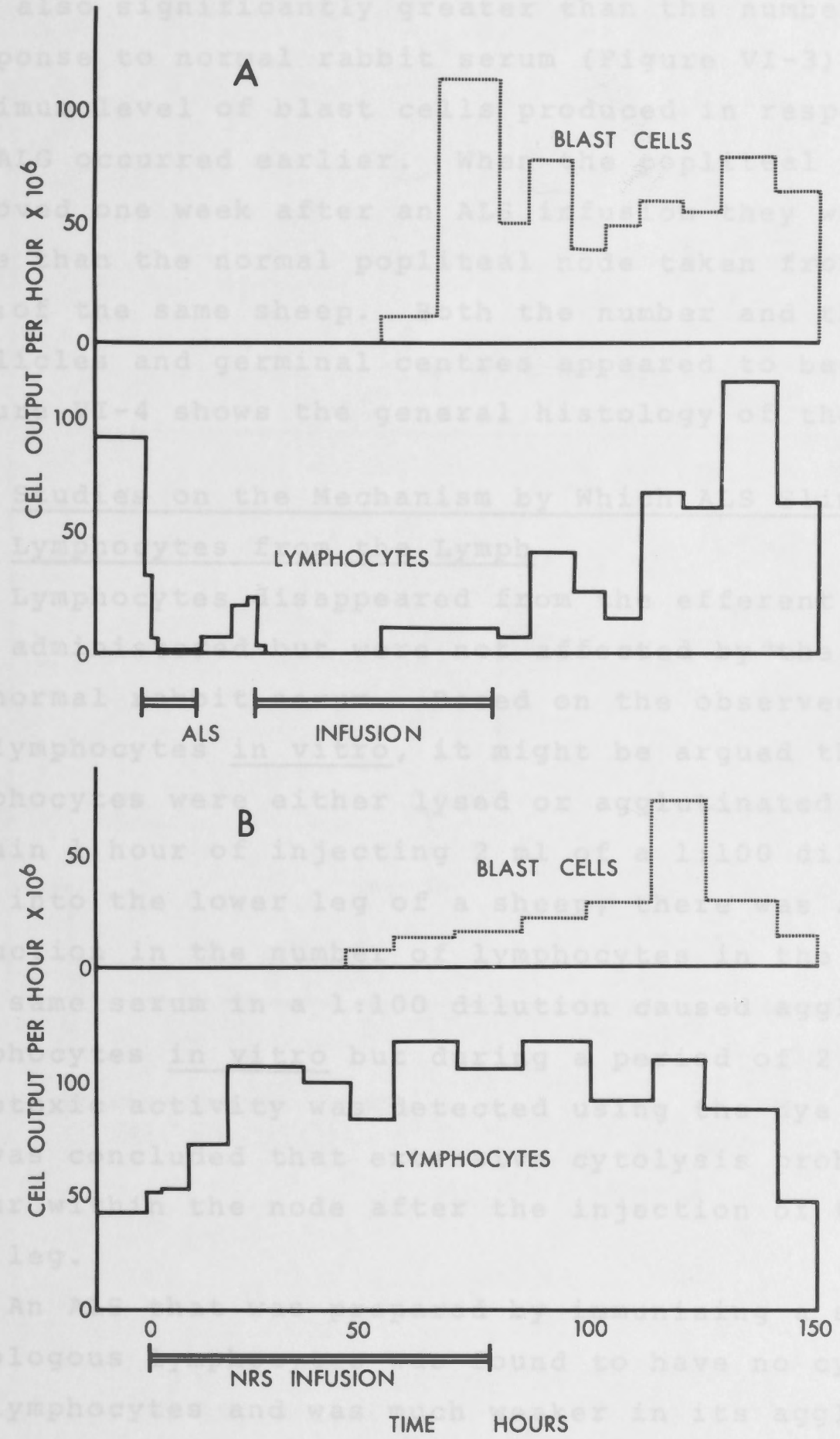
RESULTS

The effects of continuous infusions of ALS or normal rabbit serum on the cell output from the popliteal lymph node are shown in Figure VI-2. The infusion of normal rabbit serum resulted in a typical primary immune response in which there was an increase in the total cell output and in the output of blast cells, reaching a maximum at about 4-5 days. The infusion of ALS on the other hand caused a severe reduction in lymphocyte output, similar to the effect produced by a single subcutaneous injection except that the lymph remained depleted of lymphocytes for 3 days. When the infusion of ALS was stopped during the period from 15-24 hours, the cell output immediately started to recover and it was apparent that the continued presence of ALS in the node was necessary to prevent the appearance of lymphocytes in the efferent lymph. Although normal lymphocytes were eliminated from the lymph, blast cells subsequently appeared in large numbers even though the infusion was continuing. These cells, typical of those seen in immune responses, could be obtained almost as a pure population in the efferent lymph by this technique.

The Antigenicity of Rabbit Serum Proteins

As would be expected, rabbit serum was antigenic in the sheep. The amount of protein infused in the ALS experiment (Figure VI-2) was less than that infused in the normal rabbit serum experiment; the ALS however, caused a much

FIGURE VI - 2



The effects of a continuous infusion of rabbit sera on the cellular output of the popliteal lymph node.

A. Rabbit anti-lymphocyte serum.

B. Normal rabbit serum.

greater production of blast cells. The number of blast cells produced in response to the gamma-globulin fraction of ALS was also significantly greater than the number produced in response to normal rabbit serum (Figure VI-3) and the maximum level of blast cells produced in response to ALS or ALG occurred earlier. When the popliteal nodes were removed one week after an ALS infusion they weighed 3-4 times more than the normal popliteal node taken from the opposite legs of the same sheep. Both the number and the size of the follicles and germinal centres appeared to be increased. Figure VI-4 shows the general histology of these nodes.

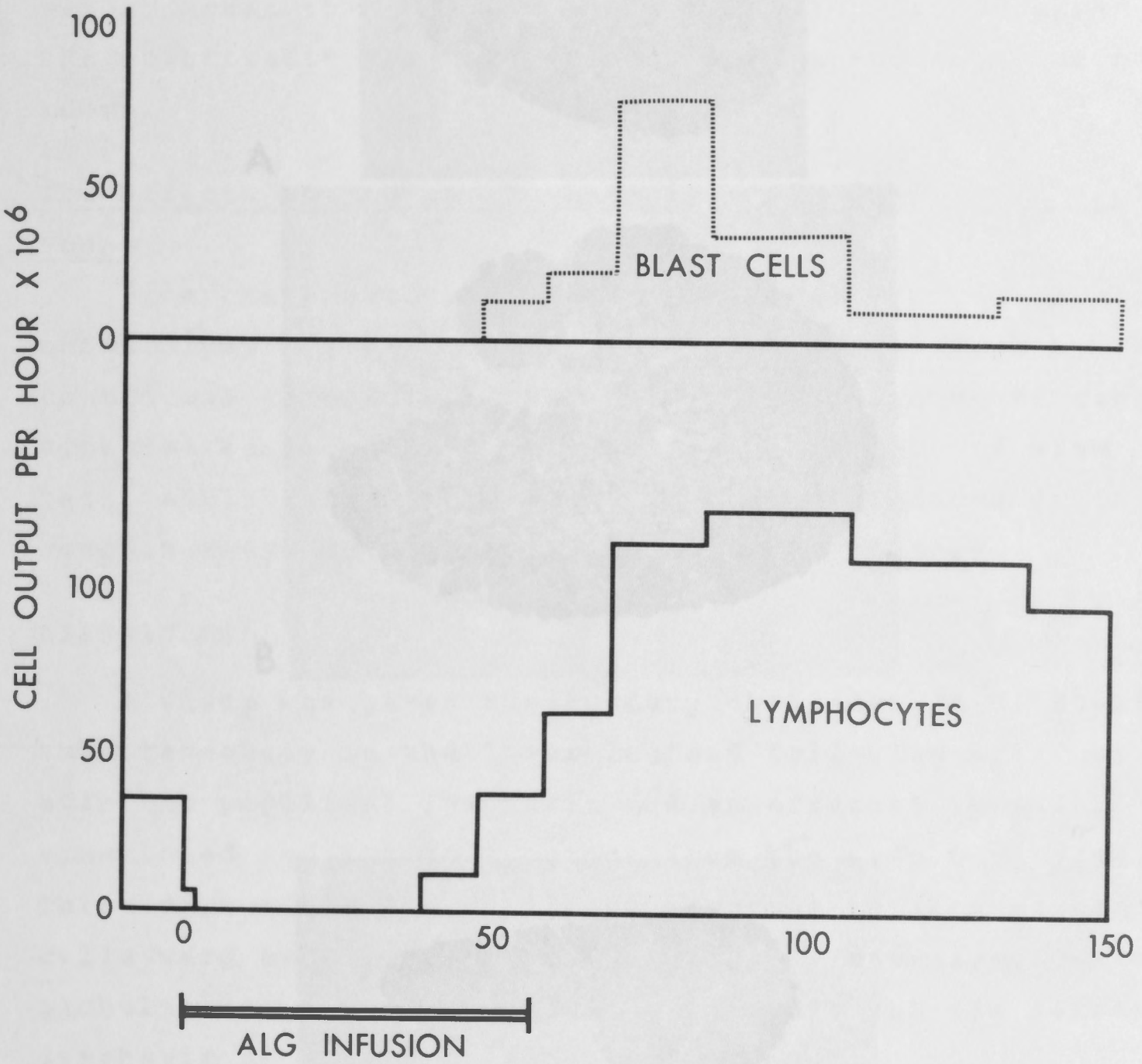
Studies on the Mechanism by Which ALS Eliminates Lymphocytes from the Lymph

Lymphocytes disappeared from the efferent lymph when ALS was administered but were not affected by the administration of normal rabbit serum. Based on the observed action of ALS on lymphocytes *in vitro*, it might be argued that the lymphocytes were either lysed or agglutinated within the node. Within 1 hour of injecting 2 ml of a 1:100 dilution of ALS into the lower leg of a sheep, there was a pronounced reduction in the number of lymphocytes in the popliteal lymph. The same serum in a 1:100 dilution caused agglutination of lymphocytes *in vitro* but during a period of 2 hours, no cytotoxic activity was detected using the dye exclusion test. It was concluded that extensive cytolysis probably did not occur within the node after the injection of the ALS into the leg.

An ALS that was prepared by immunizing a sheep with homologous lymphocytes was found to have no cytotoxic effect on lymphocytes and was much weaker in its agglutinating capacity than heterologous ALS. It was however, capable of suppressing the lymphocyte output of the popliteal node (see Chapter VII). These results suggested that lymphocytes were probably not destroyed within the node by any direct cytotoxic action of the ALS and probably not held in the node by agglutination.

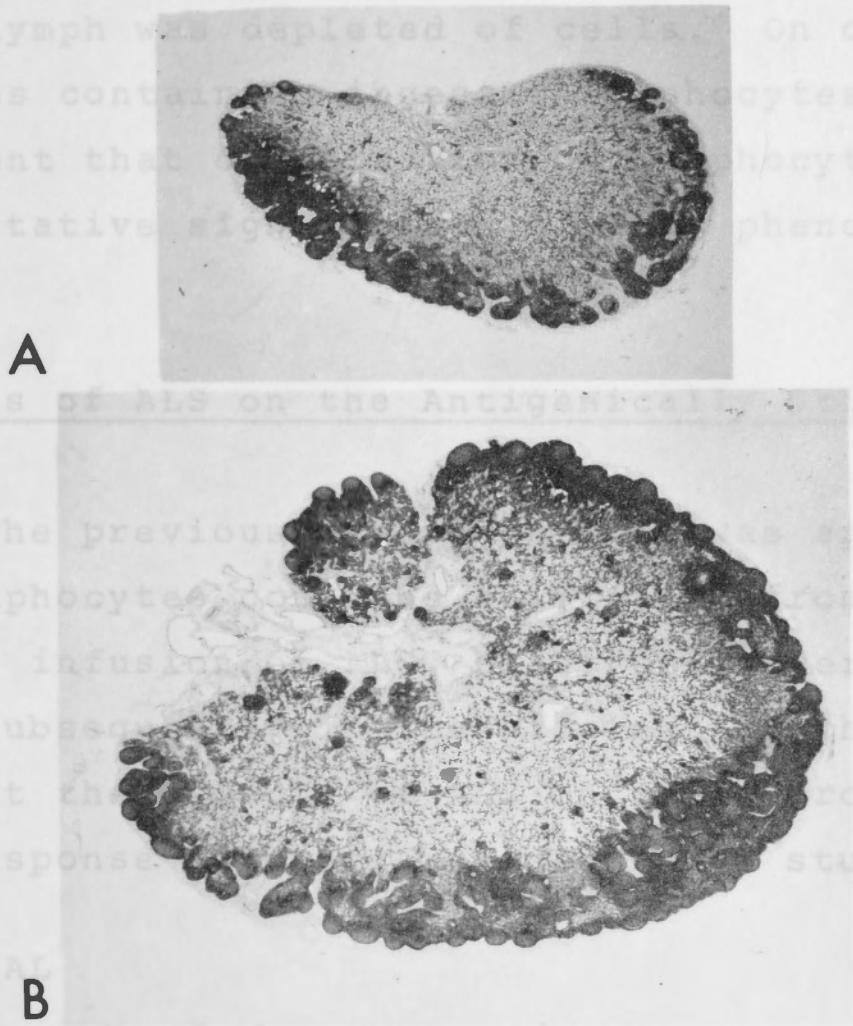
Some evidence was found which supported the idea that a mechanism involving phagocytosis was responsible for the

FIGURE VI - 3



The effects of a continuous infusion of anti-lymphocyte globulin on the cellular output of the popliteal lymph node.

FIGURE VI - 4



The appearance of popliteal lymph nodes 1 week after the infusion of ALS. Magnification X 4

A.,C. Control nodes.

B.,D. Treated nodes.

depletion of lymphocytes. Figure VI-5 shows examples of cells that sometimes appeared in the lymph during the period when the lymph was depleted of cells. On occasions macrophages containing ingested lymphocytes were seen. It was apparent that opsonization of lymphocytes occurred but the quantitative significance of this phenomenon was not known.

The Effects of ALS on the Antigenically Stimulated Popliteal Node

From the previous experiments it was apparent that whilst normal lymphocytes could be eliminated from lymph by the continuous infusion of ALS, blast cells were unaffected and appeared subsequently in the efferent lymph. In view of this result the effects of ALS on cells produced in the node in response to other antigens were studied.

EXPERIMENTAL

A sheep was given a secondary challenge of Salmonella subcutaneously in the lower leg and following this the efferent popliteal lymphatic and an afferent lymphatic were cannulated and the cell output from the node monitored. Three days after the challenge when the antibody-forming cells were at a level of 6,735 PFC/10^6, anti-lymphocyte globulin (ALG) was infused into the node via the afferent lymphatic at a rate of about 0.5 ml/hour.

RESULTS

The results of this experiment are given in Table VI-4. As before, the lymphocyte output dropped to a level of less than 1 per cent of the preinfusion levels within an hour or so of the start of the infusion and the output of PFC's appeared to be suppressed during the first few hours. The lymph flow rate and output of polymorphonuclear cells did not change significantly during the infusion. From 78-93 hours after the injection of Salmonella, the cell population of the efferent lymph was composed of over 90 per cent blast cells and the level of PFC's was maintained at over

FIGURE VI - 5

A

B

A. A macrophage in close association with a lymphocyte. Cells were obtained from efferent popliteal lymph 3 hours after the start of an ALS infusion. Magnification x 16,000.

B. A lymphocyte within a macrophage. Leishman stain. Magnification x 1,000.

TABLE VI-4

					Differential White Cell Counts					
Time after antigen hr	Flow Rate ml/hr	Total cell output/hr	Large Cells %*	ALG infusion rate ml/hr	Lympho-cytes %	Blast cells %	Neutro-phils %	Mitotic figures %	PFC/10^6	PFC/hr x 10^3
70.5-71.5	2.0	4.18×10^7	28.8	0	89.1	11.7	0	0.2	6,735	281.5
71.5-72.5	2.2	7.35×10^7	36.9	0.45	-	-	-	-	4,977	365.8
72.5-73.5	3.5	9.10×10^6	45.2	0.45	77.8	21.8	0.1	0.3	16,407	149.3
73.5-74.5	3.1	8.25×10^5	86.1	0.45	6.7	89.5	0.9	2.9	38,331	31.6
74.5-78	3.1	4.62×10^5	79.4	0.45	13.4	81.1	3.3	2.2	27,804	12.8
78-81.5	3.4	2.98×10^6	95.1	0.45	1.9	93.5	2.4	2.2	125,869	375.1
81.5-93	3.2	2.08×10^7	93.1	0.45	0.6	96.5	2.3	0.6	103,775	2,158.5
93-98	3.0	2.13×10^7	88.5	0.20†	1.1	93.6	1.5	3.8	94,746	2,018.1
98-117	1.9	2.24×10^7	60.6	0	-	-	-	-	30,333	679.5

* Coulter counter

† Infusion stopped at 94 hrs.

The effects of a continuous infusion of ALG on the cellular response of the popliteal lymph node to Salmonella.

$100,000/10^6$ cells or about 10 per cent of the total population. From the number of PFC/10^6 cells and the total cell output, it was possible to calculate the total number of antibody-forming cells released from the node. During the time interval 48-166.5 hours after antigen this amounted to 56.15×10^6 PFC.

Several experiments were done to measure the immune response to Salmonella when ALS was administered either before the injection of the antigen, at the same time as the antigen was given, or at various times after giving the antigen. These results are shown in Table VI-5 where the total number of antibody-forming cells released by the popliteal node during the entire response has been compared in each case. Although there was considerable variation between animals the results led to the conclusion that these treatments with ALS had no immunosuppressive effect, at least in so far as the production of antibody-forming cells was concerned. The differences between the PFC responses in ALS treated and untreated sheep were not statistically significant.

If the ALS was causing lymphocytes to aggregate within the lymph node and in this way preventing their release into the efferent lymph, it seemed possible that other cells might also be prevented from leaving the node by the aggregation of lymphocytes. Acute inflammatory responses were examined in conjunction with ALS administration to see more precisely the effects that ALS had on the migration of polymorphonuclear cells through the lymph node.

EXPERIMENTAL

The injection of Salmonella into the leg of a sheep resulted in an inflammatory response which was augmented by injecting ALS as well at 12 hour intervals. The migration of neutrophils from the node was followed during this reaction. In another experiment an antigenic solution from a hydatid cyst of Echinococcus granulosus was injected into the lower leg of a sheep to produce an acute inflammatory reaction in the node and this response was also followed in the efferent lymph.

TABLE VI-5

EXPERIMENT	PRIMARY RESPONSE (CONTROLS)
1	12.85×10^6 PFC
2	10.79×10^6 PFC
3	35.42×10^6 PFC
	Mean 19.69
	PRIMARY RESPONSE (ALS TREATED NODE)
4	15.96×10^6 PFC (antigen given 3 days after a 55 hr continuous ALS infusion)
	SECONDARY RESPONSE (CONTROLS)
5	24.60×10^6 PFC
6	28.91×10^6 PFC
7	19.69×10^6 PFC
8	82.28×10^6 PFC
	Mean 38.87
	SECONDARY RESPONSE (ALS TREATED NODES)
9	45.14×10^6 PFC (antigen and 2 ml of ALS injected at the same time plus 2 ml of ALS injected at 12 hour intervals until 84 hours)
10	13.09×10^6 PFC (2 ml of ALS injected every 12 hours between 48 - 96 hours after antigen)
11	56.15×10^6 PFC (ALS infused between 71.5 - 94 hours after antigen)
	Mean 38.13

The number of plaque-forming cells released by the popliteal lymph node after stimulation with Salmonella in conjunction with ALS administration.

RESULTS

Table VI-6 shows the nature of the cells appearing in the lymph during the Salmonella response. The lymphocyte output of the node was severely depressed but the neutrophils continued to circulate in large numbers, apparently increasing after each injection of ALS into the lower leg. During the period from 2 - 35.5 hours more than 10^9 neutrophils were collected in the lymph as an almost pure population. These neutrophils were not lysed by the action of ALS and complement when treated *in vitro* (Table VI-7).

A predominantly eosinophil response resulted from the injection of the parasite antigens. Between 10-11 hours after the injection of antigen in conjunction with ALS, the population of cells in the lymph was composed of 94 per cent eosinophils, 4 per cent neutrophils and 2 per cent lymphocytes. A normal immune response followed these early reactions with the production of large numbers of lymphoid blast cells.

The Cytotoxic Action of ALS on Blast Lymphoid Cells

The blast cells that appeared in the lymph during the administration of ALS were apparently unaffected by the ALS as judged both in terms of their morphology (Figure VI-6) and by their ability to release antibody in the plaque assay. This suggested that possibly the blast cells may have had differences in surface antigenicity when compared to the normal lymphocytes, i.e. that the ALS did not combine with the blasts. Such a possibility was tested *in vitro* by comparing the cytotoxic action of ALS on a relatively pure population of blast cells with its action on lymphocytes, both cell populations being obtained from the same animal.

EXPERIMENTAL

Normal efferent popliteal lymph cells were collected from one leg of a sheep while at the same time the opposite leg was challenged with Salmonella and given a continuous infusion of ALS via an afferent lymphatic. A population of cells containing 88 per cent blast cells was obtained from

TABLE VI-6

Time After Injection of Salmonella (hr)	Flow Rate ml/hr	Total Cell Output/hr	Differential White Cell Counts			
			Lympho-cytes %	Blast Cells %	Neutro-phils %	Eosino-phils %
Before	3.9	1.26×10^7	99.1	0.9	0	0
*0-11.5	13.7	2.07×10^6	35.3	1.9	49.6	13.2
*12-20	24.0	2.54×10^7	1.0	0	95.1	3.9
20-23.5	21.4	1.24×10^8	0.4	0	99.6	0
23.5-24	19.5	2.40×10^8	1.0	0	99.0	0
*24-35.5	22.5	4.46×10^7	0.4	0	99.6	0
35.5-36	18.0	7.54×10^7	3.1	0.5	96.4	0
*36-40	25.8	1.30×10^7	8.8	1.4	89.6	0.2
40-43	26.0	2.44×10^7	5.2	1.1	93.7	0

* 2 ml ALS injected into lower leg

The number of neutrophils appearing in efferent popliteal lymph during the administration of ALS.

TABLE VI-7

Cells	Treatment	Percentage of Lymphocytes Viable	
		30 min	60 min
Lymphocytes	Normal rabbit serum	> 95	> 87
	Anti-lymphocyte serum	52	5
Blast Cells	Normal rabbit serum	> 95	> 95
	Anti-lymphocyte serum	50	1
Neutrophils	Anti-lymphocyte serum	-	> 95

A comparison between the cytotoxic activity of ALS on lymphocytes, blast cells and neutrophils. Cells were incubated at 37^{o}C in the presence of complement.

FIGURE VI-6

Cells obtained from efferent lymph during a continuous, 3 day infusion of ALS into the lymph node. The cells have been stimulated by the ALS.

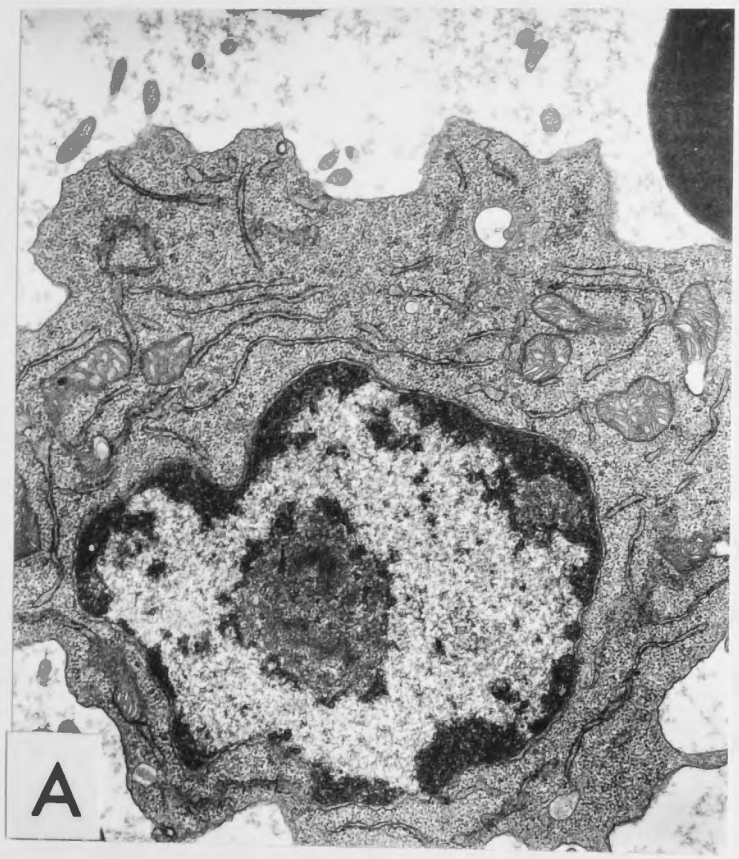
A. Blast cell.

B. Blast cell.

C. Blast cell with dilations of the ergastoplasm presumably filled with antibody.

D. Plasma cell.

Magnifications X 1100.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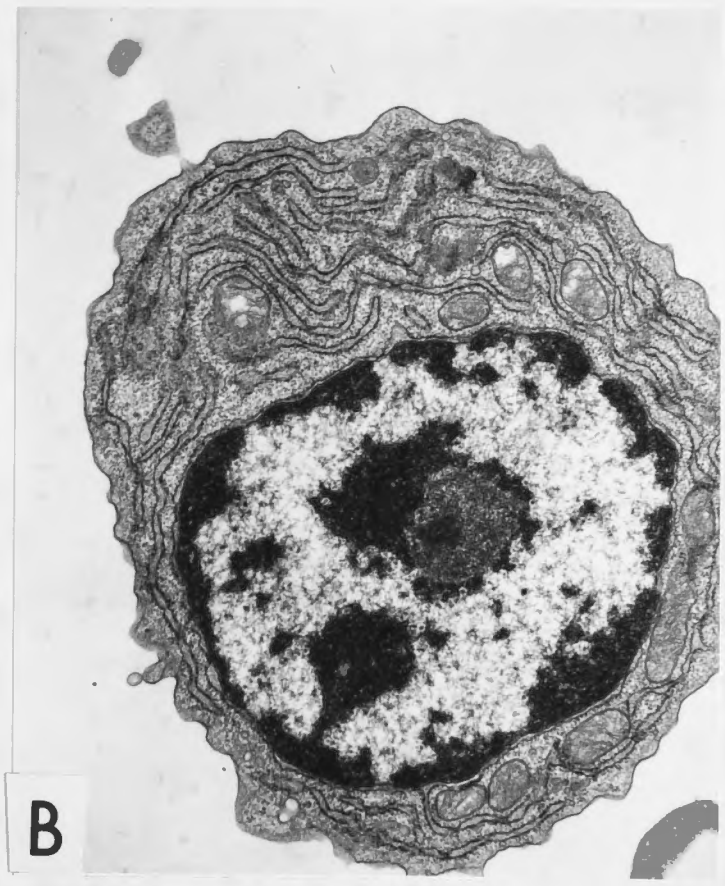
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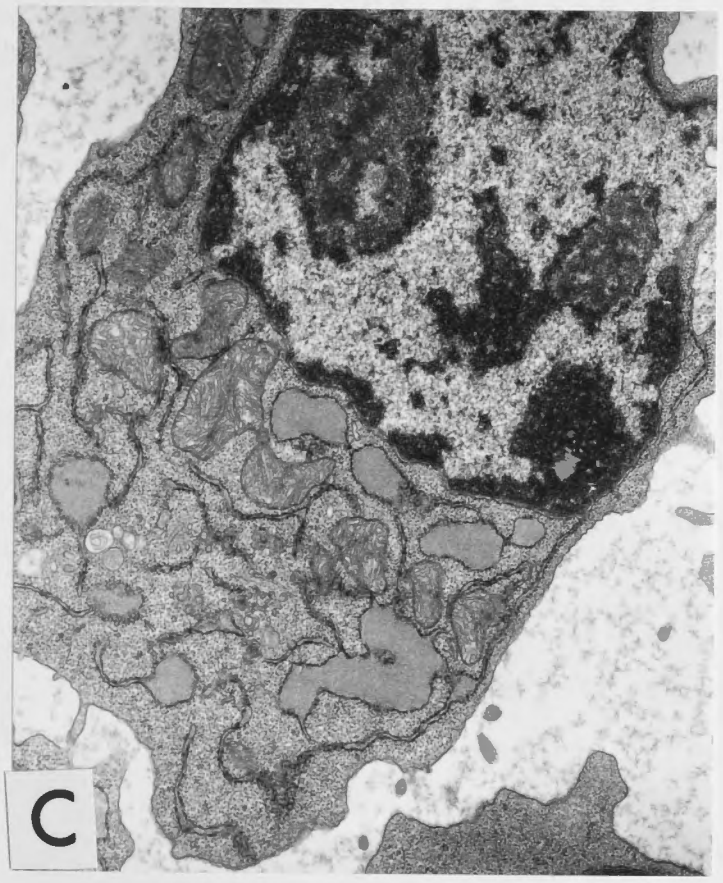

C


D

the node infused with ALS and this population was compared with the population of normal lymphocytes from the other leg.

RESULTS

Table VI-7 shows that both of these cell types were destroyed by ALS in vitro in the presence of complement. Blast cells collected during responses to swine influenza virus and homologous lymphocytes as well as the Salmonella blasts were also killed rapidly when exposed to ALS under these conditions. While both lymphocytes and blast cells were killed by the ALS the polymorphonuclear cells were not. Therefore, another reason was sought to explain the lack of effect by ALS in vivo against blast cells within the node and in the efferent lymph.

It was concluded that the ALS administered either via an efferent lymphatic directly or by subcutaneous injection prevented the traffic of lymphocytes through the node but that the ALS did not come in contact with those regions of the node where blast cells were proliferating. It was thought that the ALS was probably absorbed out by the recirculating lymphocytes in the cortico-medullary regions of the node and by other lymphocytes in the cortex. A 1ml sample of ALS was found to lose most of its activity against lymphocytes after a 1 hour incubation with 2×10^8 lymphocytes at 37°C due presumably to its absorption onto the surface of the cells.

Discussion

The preparation of an antiserum directed against lymphocytes is not a recent development. Pappenheimer (1917) produced an antiserum in rabbits against rat thymus cells and demonstrated that it agglutinated lymphocytes and in the presence of complement, lysed them. The production of heterologous antiserum against other cells dates as far back as Metchnikoff (1899) (cited by Woodruff, 1969). Chew and Lawrence (1937) demonstrated a fall in the level of blood lymphocytes following an intraperitoneal injection of

rabbit antiserum against guinea-pig lymphocytes. Current research on ALS has followed the work of Woodruff and Anderson (1963) in which it was demonstrated that in rats, the life of skin homografts could be prolonged by injections of ALS into the recipients. Antiserum against lymphocytes however, has many biological effects which may or may not be directly related to its immunosuppressive capacity.

The Agglutinating, Cytotoxic Activity of ALS and its Capacity to Cause Lymphopoenia

In the present experiments lymphocytes treated *in vitro* with ALS were rapidly agglutinated or destroyed. This result has been reported by other workers (Abaza and Woodruff, 1966; Gray, Monaco, Wood and Russell, 1966; Iwasaki, Porter, Amend, Marchioro, Zühlke and Starzl, 1967). Woodruff, Reid and James, (1967) showed that the $F(ab)_2$ portion of anti-lymphocyte globulin which contains the antigen binding sites but not the complement binding site (Fc fragment) can only agglutinate or transform lymphocytes but cannot lyse them. This confirmed previous studies which showed that the cytotoxicity of ALS and its ability to lyse lymphocytes depended on complement fixation.

Two mechanisms have been considered in the production of peripheral lymphopoenia following ALS administration. These mechanisms have been proposed in view of the fact that ALS is known to be absorbed to the surface of lymphocytes and so bring about their agglutination and lysis *in vitro*. Complement dependent lysis can be demonstrated *in vitro* but there is little evidence that this reaction occurs to any widespread extent in the animal, although Agnew (1968), reported that lymphocytes which took up trypan blue appeared in the thoracic duct of rats that had been injected with ALS. The removal of dead, damaged or ALS coated lymphocytes by the reticuloendothelial system appears to be the principal cause of the lymphopoenia. Studies have demonstrated this by using histological methods (Turk and Willoughby, 1967; Taub and Lance, 1968a) and also by studying the fate of radioactively labelled lymphocytes after treatment of the

cells themselves with ALS or by giving ALS to the animal (Martin and Miller, 1967, 1968; Denman, Denman and Embling, 1968; Denman and Frenkel, 1968; Taub and Lance, 1968b).

Medawar (1969) and others (Levey and Medawar, 1967; Taub and Lance, 1968b) favour the evidence that the lymphocytes in the blood are the immediate target of ALS rather than the lymphocytes within fixed lymphoid tissues. However, the route of administration of the ALS, its potency and the dosage used are important features which determine the nature of the effects and direct comparisons of the results obtained with different experimental protocols are not valid.

Agnew (1968) reported a significant drop in the level of lymphocytes in the thoracic duct of rats within 4 hours of injecting ALS subcutaneously at a dosage of 1.5 ml/100g body weight, a dose commonly used with small animals. Taub and Lance (1968a) found that the level of lymphocytes in the blood was reduced to 10-20 per cent of normal within 48 hours of giving a subcutaneous injection of 0.5 ml of ALS in mice. These doses of ALS in small animals have profound systemic effects. In the present experiments using sheep and giving the ALS subcutaneously or by perfusion via an afferent lymphatic only the single popliteal node was involved. Effects were obtained in the lymph node with very small doses of ALS compared with those that have been used in small animals. On a body weight basis a comparable dose in the sheep would be about 750 ml given subcutaneously.

What happens to the 10^8 - 10^9 lymphocytes that fail to leave the popliteal node in the lymph during the 10 hours or so following injection of ALS has not been determined conclusively. However the results obtained do serve to limit the possible explanations. The elimination of lymphocytes from the efferent lymph is very rapid, beginning within minutes of starting the ALS infusion or of giving the subcutaneous injection. This seems too rapid for extensive cytolysis to have occurred particularly in view of the rate at which ALS destroyed lymphocytes <u>in vitro</u>. Phagocytosis however, induced by ALS acting as an opsonizing agent could

probably occur within this space of time and as stated, occasional macrophages containing lymphocytes were sometimes observed in the efferent lymph soon after the injection of ALS. Another possible way in which ALS may act could be by preventing the recirculation of lymphocytes through the post-capillary venules. However, lymphocytes that enter the node via the afferent lymphatics and which can contribute up to 10 per cent of the efferent cells (Hall and Morris, 1965a) are also suppressed by ALS treatment because the cell output is usually depleted by more than 99 per cent. The continued egress of polymorphonuclear cells from the node in large numbers during the period in which the lymphocyte output is reduced seemed incompatible with any physical blockage of lymphatic sinuses due to aggregated lymphocytes. Many of the polymorphonuclear cells were probably leaving the blood to enter the lymphatics at the injection site in the lower leg (Smith and Morris, 1970; Chapter IX, this thesis) as well as within the lymph node. Marchesi and Gowans, (1964) claimed that polymorphonuclear cells also migrate through the post-capillary venules in lymph nodes. Therefore, the passage of cells through the substance of the node by way of either the subcapsular sinus or the medullary sinuses seemed to be unimpeded. Removal of ALS coated lymphocytes by phagocytic cells remained the only explanation for which any positive evidence was found in the present experiments to explain the fate of these cells.

The Capacity of ALS to Induce Cell Transformation

The transformation of lymphocytes in culture can be brought about by two general classes of agents. Antigens can effect lymphocyte transformation *in vitro* but only if the lymphocytes have been obtained from animals or humans previously immunized against the specific antigens. The other class of agents act on normal, unsensitized lymphocytes and includes the so-called, non-specific mitogens eg. phytohaemagglutinin, pokeweed mitogen, and ALS. Homologous lymphocytes are generally thought to stimulate unsensitized lymphocytes due to the different histocompatibility antigens

on their surface (Ling, 1968) but other mechanisms have also been considered to play a role (Lafferty and Jones, 1969).

In the experiments described here normal rabbit serum was not capable of inducing the transformation of sheep lymphocytes in culture although it was antigenic when administered directly into the animal. ALS on the other hand was found to transform lymphocytes in culture in agreement with the findings of other workers (Grasbeck, Nordman and de la Chapelle, 1964; Holt, Ling, and Stanworth, 1966; Woodruff, Reid and James, 1967; Foerster, Lamelin, Green, and Benacerraf, 1969) and it also gave rise to a larger blast cell response than normal rabbit serum when tested in vivo. The hypertrophy of lymphoid tissue following ALS treatment has already been described. Levey and Medawar (1966) observed blast cell transformation of peripheral blood lymphocytes as well as hypertrophy of lymphoid organs after ALS treatment. However, Turk and Willoughby (1967) demonstrated that the paracortical regions of guinea-pig lymph nodes were depleted of lymphocytes after ALS administration for a period of 6 days; in contrast the follicles were not affected. The lymph nodes removed from sheep after ALS perfusions were significantly enlarged and also appeared to contain an increased number of follicles. Lymphocytes in the paracortical regions of the node did not appear to be affected, due probably to the continued repopulation of this area by cells migrating from the blood.

For ALS to effect the transformation of lymphocytes in vitro complement must be absent or the lymphocytes would be lysed. As lymphocytes underwent transformation in response to ALS in vivo there must be areas within the lymph node where ALS comes into contact with lymphocytes and induces their transformation and yet the concentration of complement in such regions must be insufficient to promote cytolysis.

ALS has been called a non-specific lymphocyte mitogen. This implies that lymphocytes stimulated by ALS do not produce specific antibody. Whilst ALS stimulated cells have never been shown to produce antibody in vitro, antibody production in response to ALS in vivo has been demonstrated (Currey and

Ziff, 1966; Iwasaki et al, 1967; Clark, James and Woodruff, 1967). The stimulation of lymphocytes by ALS and not by normal serum proteins in vitro could be related to the culture conditions. It is obvious that serum proteins are antigenic in vivo even though culture assay conditions fail to detect any evidence of cell transformation. ALS may be more efficient at inducing lymphocyte transformation than normal serum proteins but there is no real evidence that it does not act as an antigen in this process.

The effect on lymphocytes of the antiserum raised against sheep serum proteins is relevant to current immunological theory. The mechanism of antigen recognition by cells is a fundamental but, as yet unsolved problem. Current theoretical opinion favours a receptor site hypothesis which states that lymphocytes have antigen receptors on their surface which are immunoglobulin molecules (Jerne, 1967; Mitchison, 1969). When antigen combines with the receptor this somehow acts as a trigger which induces the lymphocyte to transform. The best experimental evidence supporting this concept is the work of Sell and Gell (1965) where they showed that antiserum directed against a rabbit immunoglobulin allotype could stimulate blast transformation of allotype-specific rabbit lymphocytes. Antisera against a range of rabbit immunoglobulins and immunoglobulin chains and fragments were also capable of transforming lymphocytes (Sell, 1967a; Sell, 1967b). It is important that these experiments be repeated in other species and situations before it can be considered as proof for the existence of immunoglobulin receptor sites. It would seem probable on the basis of this theory that an antiserum directed against a wide spectrum of immunoglobulin molecules (receptor sites) would activate a large number of lymphocytes, although Sell (1967a) was unable to demonstrate any summation effects by combining antiserum against more than one immunoglobulin class. In the experiments described in this Chapter rabbit anti-sheep serum did not stimulate sheep lymphocytes in vitro under conditions of culture which were adequate to allow lymphocytes to transform in the presence of ALS. There have

been no reports of lymphocyte stimulation by anti-immunoglobulin sera in species other than the rabbit.

ALS and Immunosuppression

Although there is evidence that ALS suppresses the immune response to some antigens in rodents (Monaco, Wood, Gray and Russell, 1966; James and Anderson, 1967; James and Jubb, 1967; Martin and Miller, 1968), a variety of humoral antibody responses, particularly secondary responses are not affected and ALS is considered to act principally as a suppressant of so-called, cell-mediated immune responses such as the homograft reaction (Woodruff, 1967). In homograft situations evidence has been presented which claimed to show that the immunosuppressive capacity of ALS was not related to its ability to produce lymphopoenia in the blood (Jeejeeboy, 1967). Other experiments however, have shown a positive correlation between lymphopenia and homograft survival (Russell and Monaco, 1967). The actual traffic of lymphocytes from blood to lymph through a homograft may be an important factor in graft rejection (Pedersen and Morris, 1970) and the extent of such traffic may not always be directly related to the number of lymphocytes in the blood stream. For example, where a homograft is "well matched" with the recipient this may not induce a large scale traffic of cells from the blood stream through the graft; in this instance the number of cells entering the graft would be independent of the actual number of circulating blood lymphocytes.

Medawar (1969) concluded that ALS probably acts by selectively depleting lymphocytes belonging to the recirculating, long-lived, thymus-dependent pool. The evidence for this is based on the histological appearance of lymphoid tissue after ALS treatment which shows lymphocyte depletion in the "thymus dependent" paracortical region of lymph nodes (Parrott, de Sousa, and East, 1966). In addition the effects of ALS are most dramatic in relation to the cell-mediated responses, which also appear to have an element of "thymus dependency" (Miller and Osoba, 1967). Martin et al (1968),

in studies based on cooperation between bone-marrow and thymus cells, suggested that ALG acts selectively on thymus derived, "antigen-reactive" cells by opsonization and removal by the reticulo-endothelial system. Möller and Zukoski (1968) used an irradiated-recipient transfer model and also claimed that ALS preferentially inactivated only antigen-sensitive cells and they suggested that these cells were physically different from antibody-forming cells.

The present experiments showed that both antibody-forming cells (blasts) and lymphocytes were lysed by ALS and complement in vitro. In fact virtually all of the lymphocytes or blasts taken from lymph were destroyed by ALS after 1 hour. When ALS was administered in vivo however, only the lymphocytes leaving the node via the efferent lymph and not the blast cells were affected and no suppression of the numbers of plaque-forming cells was found during the immune responses to Salmonella. It was concluded that the small lymphocytes and the blast cells were in separate compartments of the node and that the recirculating small lymphocytes were absorbing most of the ALS administered to the node. Because of this the concentration of ALS in the vicinity of the blast cells, which are found predominantly in the medullary cords, was reduced. It is not necessary and in fact almost certainly incorrect to postulate that this difference in action of ALS is related to whether or not the cells originated in the bone-marrow or the thymus.

Purification of Cells Using ALS

The administration of ALS during an immune response proved to be a useful method for obtaining large numbers of specific, antigen-stimulated cells in almost pure populations. These cells could be recovered in the lymph in good condition, apparently unaffected by the ALS. This was illustrated by the high specific activity of antibody-forming cells obtained in the lymph in response to Salmonella (125,869/10^6 cells) and the high proportion of blast cells (over 90 per cent). If this procedure had been used during an immune response to horse-radish peroxidase presumably some 70-80 per cent of

the total cells in lymph would have contained detectable antibody.

By using ALS to prevent lymphocyte circulation, large numbers of virtually pure populations of neutrophils or eosinophils could also be obtained.

Summary

1. An antiserum was prepared in rabbits against cells from the efferent popliteal lymph of sheep. This ALS was capable of destroying, agglutinating, and transforming sheep lymphocytes *in vitro*.

2. Local administration of this ALS in low dosage was sufficient to almost completely eliminate recirculating lymphocytes from the efferent popliteal lymph.

3. Cells produced within the node in response to ALS or other antigens were not affected by ALS that was administered *in vivo* and these cells appeared in the efferent lymph even though they were destroyed by the action of ALS *in vitro*.

4. The antibody-forming cell response of the popliteal node to Salmonella could not be reduced by ALS.

5. ALS was demonstrated to be useful in purifying large numbers of antigen-stimulated cells or polymorphonuclear cells by virtue of its capacity to eliminate the recirculating lymphocytes in the efferent lymph.

CHAPTER VII

THE EFFECT OF A LOCALIZED GRAFT-VERSUS HOST-REACTION ON THE CELLULAR AND HUMORAL ANTIBODY CONTENT OF LYMPH

The Effect of a Localized Graft-Versus-Host Reaction on the Cellular and Humoral Antibody Content of Lymph

The recirculating small lymphocyte has been implicated as the effector cell in the rejection of homografts, and in graft-versus-host (GVH) reactions. The work of Gowans and his associates (see Gowans and McGregor, 1965) involving the inoculation of labelled small lymphocytes from parental strain rats into F_1 hybrids or lethally irradiated mice confirmed previous indirect evidence that the lymphocytes repopulate the lymphoid tissues of the host. They also described the transformation of small lymphocytes into blast forms which stained intensely with pyronin stain. Further evidence implicating the lymphocyte in GVH reactions has been found in experiments carried out on a wide variety of species (see Billingham, 1966).

In the study of graft-versus-host reactions, immunologically competent cells are usually introduced into homologous recipients that themselves lack complete immunological competence. The systems used have been parental strain cells injected into either neonatal or adult F_1 hybrids (Billingham and Brent, 1957; Oliner, Schwartz and Dameshek, 1961), lymphoid cells injected into tolerant recipients (Billingham and Silvers, 1961) or lymphocytes injected into chicken embryos or inoculated onto the chorio-allantoic membrane of the egg (Simonsen, 1957). Other types of local GVH reactions can be evoked and these are characterized by lesions which form at the site of inoculation of homologous, immunologically competent cells. Elkins,(1964, 1966) has described the reaction of lymphoid cells from parental strain rats when they are injected beneath the kidney capsule of F_1 hybrid hosts. Brent, Brown, and Medawar (1962) described the normal lymphocyte transfer (NLT) reaction which develops in guinea pigs if homologous lymphocytes are injected into the skin of either unrelated or F_1 hybrid recipients. NLT reactions have also been described in chickens (Warner, 1964), hamsters (Ramseier and Billingham, 1966), rats (Ford, 1967) and sheep (Jones and Lafferty, 1969). These homologous

reactions are characterized by various degrees of either local and/or systemic tissue destruction and by various other pathological manifestations such as the enlargement of the spleen and liver.

Although the lymphocyte appears to be the effector cell in all these reactions, the mechanism producing the tissue destruction is not understood. It is not clear whether the early manifestations of the reactions are due to the "aggression" of the small lymphocyte itself against the homologous tissue or whether the transformed lymphocyte or the products released by these cells are the damaging agents.

The experiments described in this chapter were designed to examine the dynamics of the development of an NLT lesion by monitoring the changes in the afferent lymph draining from the site where the homologous cells were injected, and the efferent lymph draining from the regional lymph node. Both the cells and the homologous anti-lymphocyte antibody produced during the reaction have been studied.

Evidence for tissue destruction was based on gross and histological examination and on the changes brought about in the composition and rate of formation of lymph due to changes in permeability of the blood vessels in the area.

Results

Kinetics of the Development of an NLT Lesion

EXPERIMENTAL

Normal lymphocytes were collected from the efferent popliteal lymph of a donor sheep. These cells were injected intra-dermally into several sheep in the region of the inguinal skin where wool growth is scanty; the development of the skin lesion was followed by measuring the increase in skin thickness at daily intervals. In addition, 10^9 lymphocytes suspended in 2 ml of PBS were injected into the skin on the lateral aspect of the lower leg of a sheep in which the efferent popliteal lymphatic and one afferent

popliteal lymphatic had been cannulated prior to the injection. Several injection sites, both intradermal and subcutaneous, were used to ensure that the cannulated afferent lymphatic was delivering lymph from an area where the reaction was taking place and at the same time ensure that some of the injected cells would reach the popliteal lymph node. The output of lymph and cells was then followed over a period of two weeks.

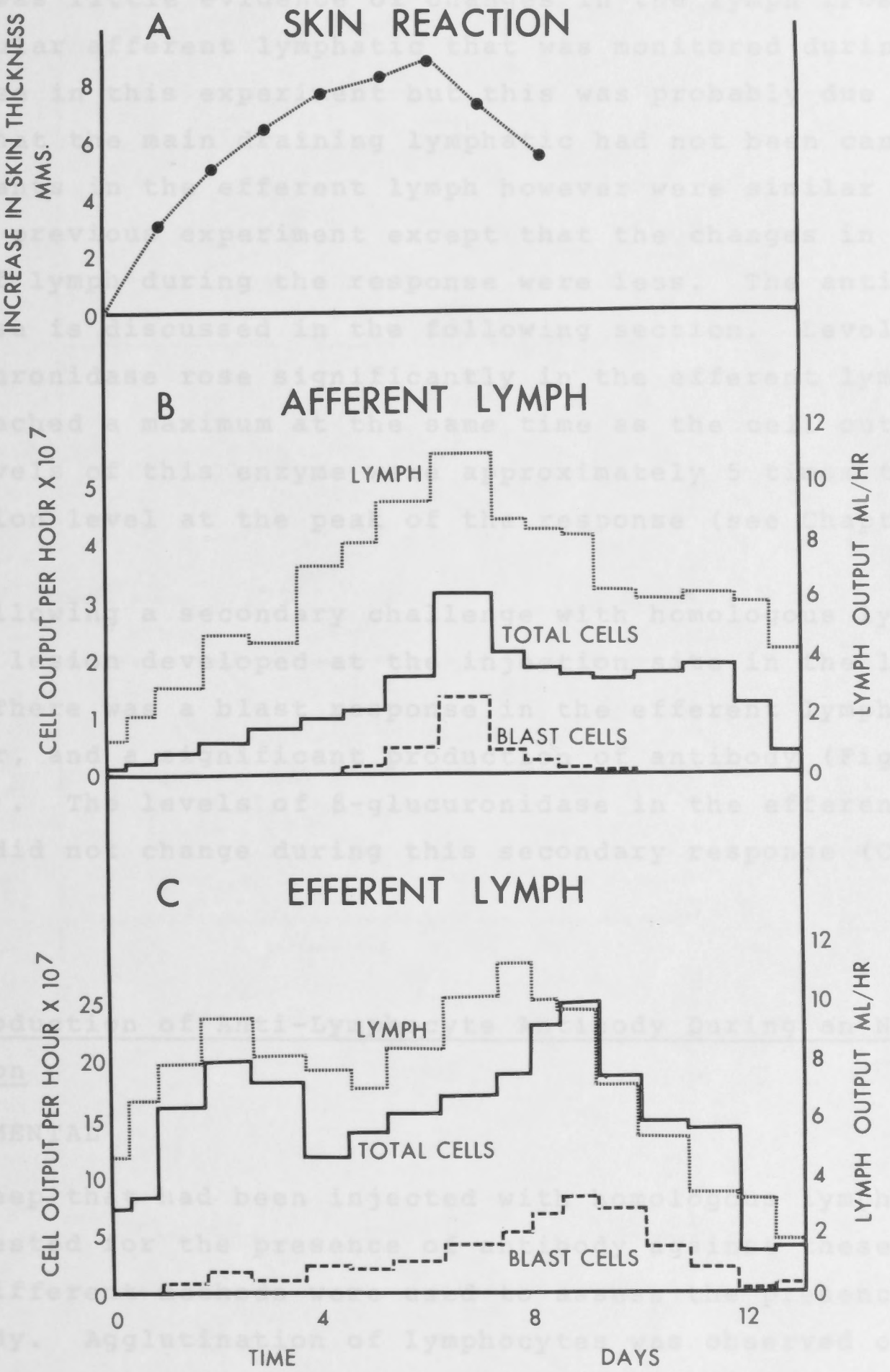
In a second experiment, a similar procedure was followed but the effects of a secondary challenge with a similar number of lymphocytes from the same donor sheep were also monitored.

RESULTS

The development of an NLT lesion in terms of the changes in skin thickness is shown in Figure VII-1 a. The skin thickness usually reached a maximum between 5 and 7 days depending on the reactivity between the donor-recipient pair of sheep and in some cases the reaction was violent enough to cause necrosis of the skin.

Figure VII-1 b and VII-1 c shows the cell output in the afferent and efferent lymph and the rate of lymph flow after the injection of the donor cells into the lower leg. The peak of the blast cell response in the afferent lymph preceded the blast cell peak in the efferent lymph by about two days. In the efferent lymph there was a phase in which normal lymphocytes were recruited into the node; this phase preceded the blast cell response from the node. In the afferent lymph no phase of cell recruitment appeared to occur and the total cell output increased as the output of the blast cells increased. The output of cells in the afferent lymph rose to a value approximately 40 times the pre-injection level, while the rate of lymph flow reached a maximum level at the same time as the cell output reached its peak. The lower leg was visibly oedematous at this time. Based on examination of lymph smears there was no involvement of polymorphonuclear cells at any stage of the response.

FIGURE VII - 1



Changes in the afferent and efferent lymph draining the site of an NLT reaction.

Figure VII-2 a shows the results of a second similar experiment in which only the efferent lymph data are shown. There was little evidence of changes in the lymph from the particular afferent lymphatic that was monitored during the response in this experiment but this was probably due to the fact that the main draining lymphatic had not been cannulated. The events in the efferent lymph however were similar to those in the previous experiment except that the changes in the flow rate of lymph during the response were less. The antibody response is discussed in the following section. Levels of β-glucuronidase rose significantly in the efferent lymph and reached a maximum at the same time as the cell output. The levels of this enzyme were approximately 5 times the pre-injection level at the peak of the response (see Chapter VIII).

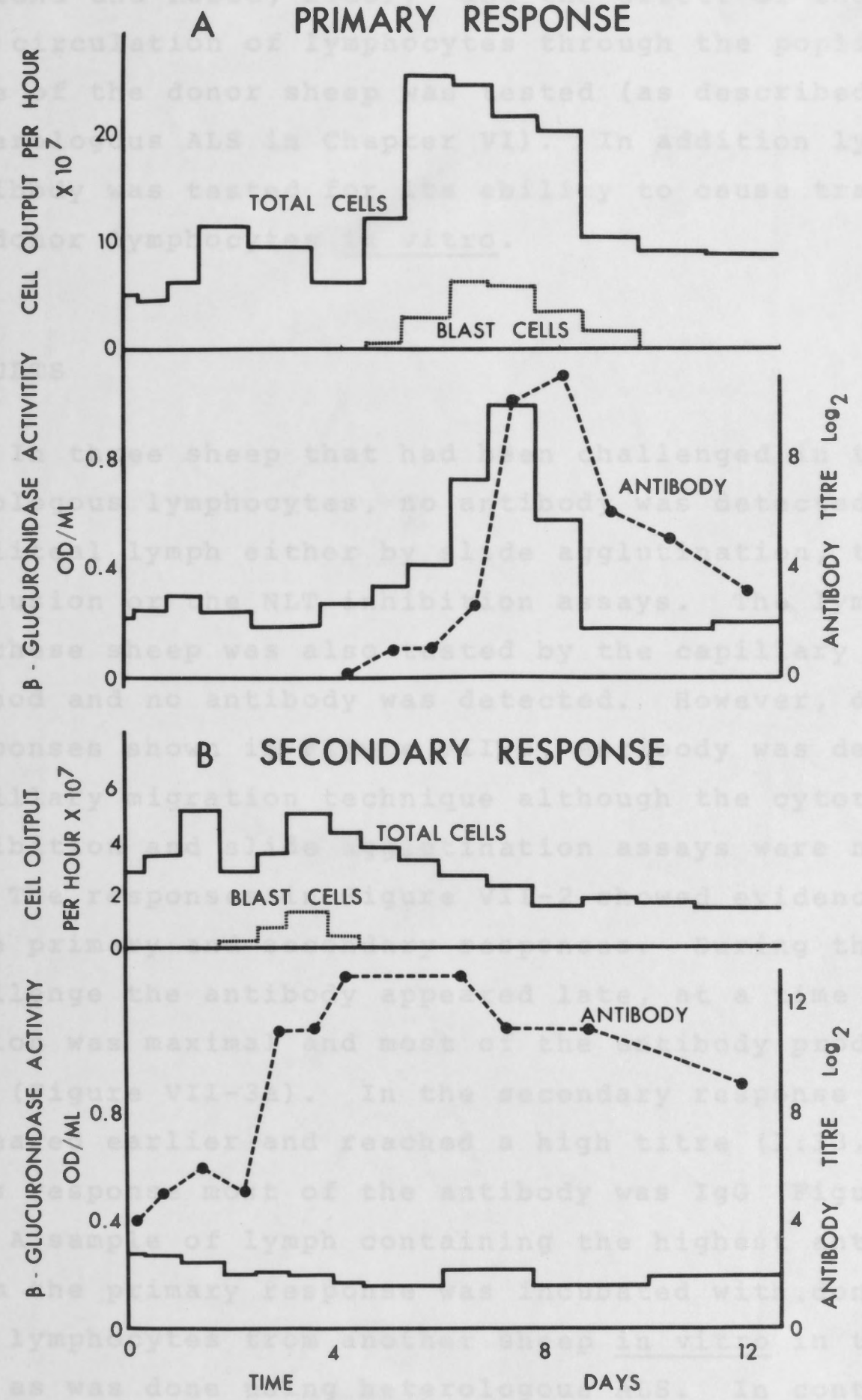
Following a secondary challenge with homologous lymphocytes, no NLT lesion developed at the injection site in the lower leg. There was a blast response in the efferent lymph however, and a significant production of antibody (Figure VII-2b). The levels of β-glucuronidase in the efferent lymph did not change during this secondary response (Chapter VIII).

The Production of Anti-Lymphocyte Antibody During an NLT Reaction

EXPERIMENTAL

Sheep that had been injected with homologous lymphocytes were tested for the presence of antibody against these cells. Five different methods were used to assess the presence of antibody. Agglutination of lymphocytes was observed on slides under the low power of the microscope; cytotoxicity was tested for by the trypan blue exclusion method in the presence of complement; lymphocytes were incubated with complement in lymph suspected of containing antibody and then the cells were subsequently tested for their capacity to produce an NLT lesion in another sheep (the effect of heterologous ALS on lymphocytes was tested in this manner in

FIGURE VII - 2



Changes in the efferent lymph during a primary (A) and a secondary (B) response to homologous lymphocytes.

Chapter VI); agglutinating antibody was measured by the capillary migration method (Thompson, Severson, Lavender, Forland and Russe, 1968); and the effect of the antibody on the circulation of lymphocytes through the popliteal lymph node of the donor sheep was tested (as described for heterologous ALS in Chapter VI). In addition lymph containing antibody was tested for its ability to cause transformation of donor lymphocytes *in vitro*.

RESULTS

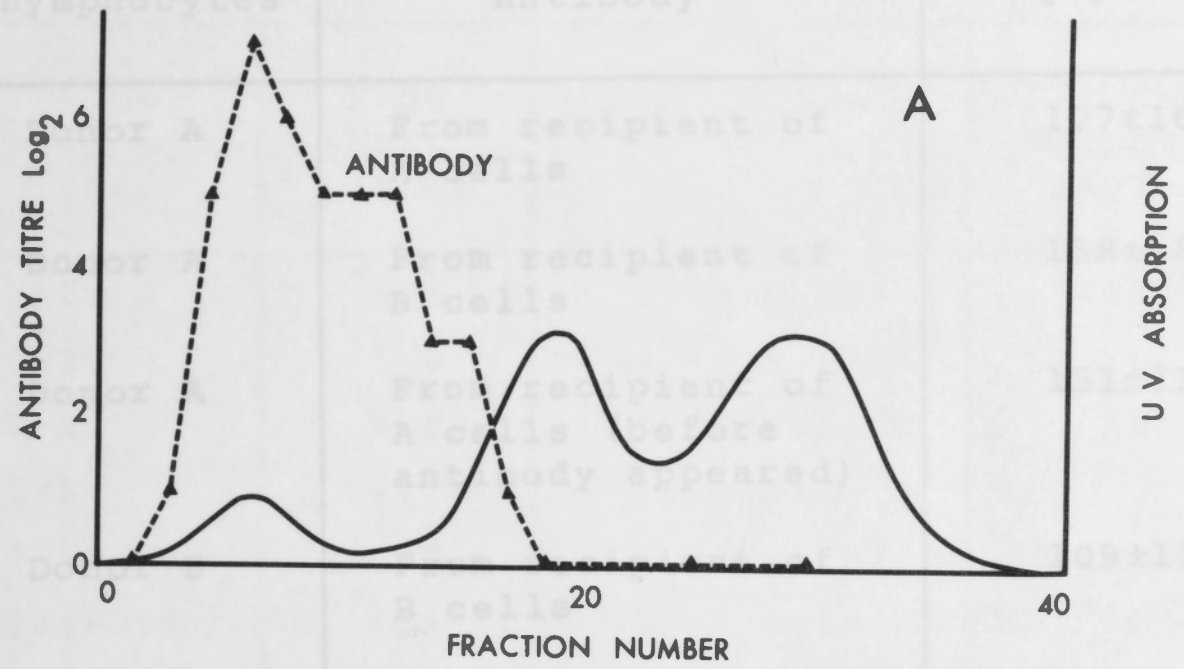
In three sheep that had been challenged in the leg with homologous lymphocytes, no antibody was detected in the popliteal lymph either by slide agglutination, trypan blue exclusion or the NLT inhibition assays. The lymph from one of these sheep was also tested by the capillary migration method and no antibody was detected. However, during the two responses shown in Figure VII-2, antibody was detected by the capillary migration technique although the cytotoxic, NLT inhibition and slide agglutination assays were negative.

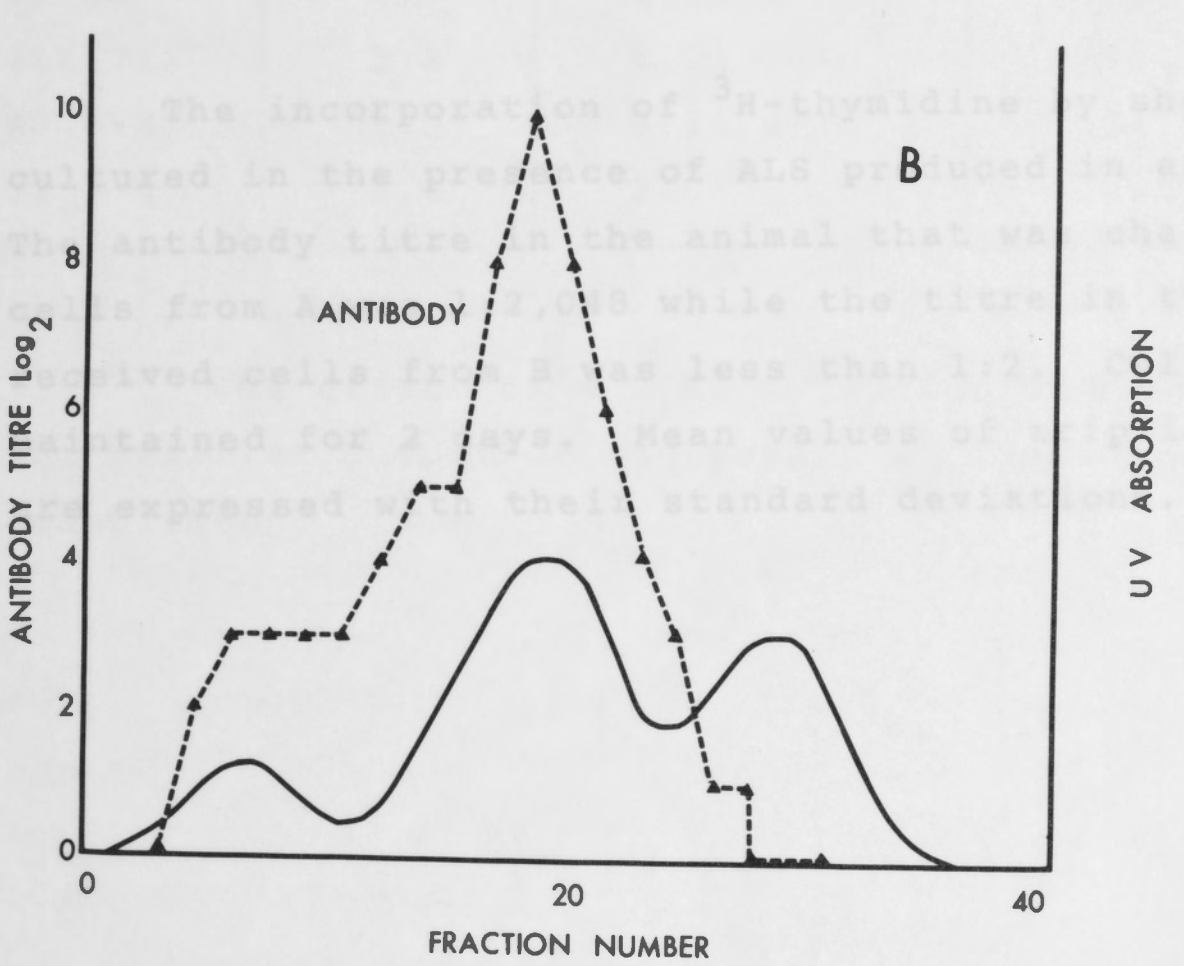
The responses in Figure VII-2 showed evidence of being true primary and secondary responses. During the primary challenge the antibody appeared late, at a time when the NLT lesion was maximal and most of the antibody produced was IgM (Figure VII-3a). In the secondary response the antibody appeared earlier and reached a high titre (1:33,000). In this response most of the antibody was IgG (Figure VII-3b).

A sample of lymph containing the highest antibody titre from the primary response was incubated with donor lymphocytes and lymphocytes from another sheep *in vitro* in the same way as was done using heterologous ALS. In contrast to the experiment with heterologous ALS, there was no evidence that homologous ALS induced lymphocyte transformation (Table VII-1).

A sample of the same lymph from the primary response, however, was capable of inhibiting the normal circulation of lymphocytes through the popliteal node of the donor sheep when it was injected into the lower leg (Table VII-2). The

FIGURE VII - 3





The separation, using Sephadex G-200 of a pooled lymph sample obtained during a primary (A) and a secondary (B) response to homologous lymphocytes.

TABLE VII-1

Source of Lymphocytes	Source of Antibody	cpm/culture
Donor A	From recipient of A cells	107±16
Donor A	From recipient of B cells	158±18
Donor A	From recipient of A cells (before antibody appeared)	151±13
Donor B	From recipient of B cells	109±12
Donor B	From recipient of B cells	133±24

The incorporation of ^{3}H-thymidine by sheep lymphocytes cultured in the presence of ALS produced in another sheep. The antibody titre in the animal that was challenged with cells from A was 1:2,048 while the titre in the animal that received cells from B was less than 1:2. Cultures were maintained for 2 days. Mean values of triplicate cultures are expressed with their standard deviations.

TABLE VII-2

Time hr	Flow Rate ml/hr	Cell Output hr	Per Cent of Output Before Injection
Before	5.5	2.35×10^7	100
0-1	6.0	3.60×10^6	15.3
1-3	4.8	4.22×10^5	1.8
3-5	7.2	2.00×10^5	0.9
5-7.5	5.7	2.37×10^5	1.0
7.5-9.5	6.5	2.22×10^5	0.9
9.5-11.5	5.5	3.71×10^5	1.6
11.5-22	4.5	2.81×10^6	12.0
22-26	4.9	1.04×10^7	44.3
26-27.5	5.2	2.24×10^7	95.3
27.5-39.5	5.0	3.15×10^7	134.0
39.5-47	4.1	2.51×10^7	106.8

The effect of a 2 ml injection of homologous ALS on the cellular output of the popliteal lymph node of the donor sheep.

lymphocyte output dropped within the first hour after a 2 ml lymph sample was injected and the lymphocyte output in the efferent lymph was reduced to less than 2 per cent of the pre-injection value for more than 12 hours. A 2 ml sample of lymph taken from the same sheep during the same response but at a time before antibody was detectable had no effect on the lymphocyte output of a node when injected into the leg. There was virtually no blast cell response subsequently in the efferent lymph following the injection of either of the lymph samples.

The properties of heterologous ALS and homologous ALS are summarized and compared in Table VII-3.

Some Properties of the Cells Collected in the Efferent and Afferent Popliteal Lymph During an NLT Response

Jones, Yamashita and Lafferty (1969) have described the histology of NLT lesions in the sheep and compared these lesions with delayed-type hypersensitivity reactions. The NLT lesions appeared to be much more invasive and to involve much larger numbers of primitive, pyroninophilic cells. Since it seemed likely that most of the cells present in the lesion were of host origin, Jones *et al* concluded that these primitive cells, once formed, were capable of damaging surrounding autologous tissue elements in a non-specific manner.

The blast cells found in both afferent and efferent lymph coming from an NLT lesion were examined and compared with the blasts that arise after challenging the node with other antigens. It was assumed that the blasts that appear in the lymph are similar to the blasts described by Jones *et al* (1969) within the NLT lesion itself.

EXPERIMENTAL

An NLT reaction was established in the lower leg of a sheep and the efferent popliteal lymphatics were cannulated on both hind legs of the animal. When the blast cells appeared in the efferent lymph they were examined for their capacity to incorporate ^{3}H-thymidine and ^{3}H-uridine by

TABLE VII-3

Homologous ALS	Assay Method	Heterologous ALS
-	Agglutination (slide)	++
+	Agglutination (Capillary Migration)	++
-	Cytotoxicity (Trypan Blue)	++
-	Cytotoxicity (NLT Inhibition)	++
-	Lymphocyte Transformation (in Vitro)	++
-	Lymphocyte Transformation (in Vivo)	++
++	Inhibition of Lymphocyte Circulation through the Lymph Node	++

A comparison of the properties of homologous and heterologous ALS.

incubating the cells for 1 hour at 37°C in Eagles' medium containing 10 percent foetal calf serum and radio-active precursors at a concentration of 1 μ Ci/ml. Afferent and efferent cells were obtained 140 hours after the injection of the lymphocytes and they were compared with normal prefemoral efferent cells from the same sheep and normal afferent popliteal cells from another sheep. In addition normal efferent lymph cells and the efferent cells containing the blasts were injected intra-dermally into the skin of the donor sheep and two other sheep to see if they were capable of producing a dermal transfer reaction. In other experiments the efferent cells were collected during an immune response to influenza virus or Salmonella and these populations of cells were compared with normal efferent cells from the same donor using the dermal transfer reaction as an assay.

RESULTS

The blast cells that appeared in the afferent and efferent lymph during an NLT reaction were similar cytologically to those described during immune responses to a variety of antigens. Cell populations containing NLT stimulated cells were much more active in the uptake of both RNA and DNA precursors (Table VII-4) than normal lymph cells. The extent of this uptake appeared to be related to the percentage of blasts in the population regardless of whether they were afferent or efferent cells although in this example cells that were not classed as blasts may have been synthesizing DNA in the afferent lymph.

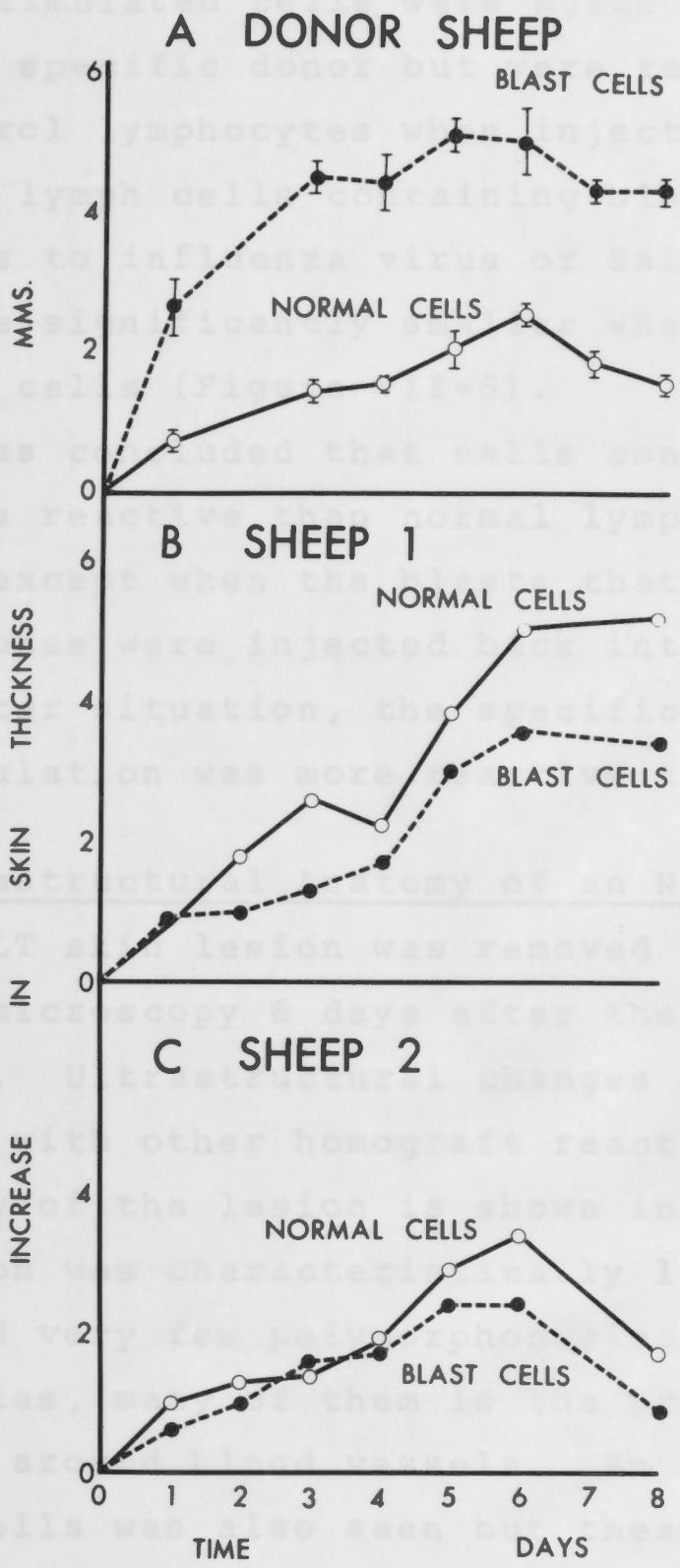
Figure VII-4 shows the result of one of the dermal transfer reaction experiments. Enhancement of the skin reaction was observed when the cells containing the blasts were injected into the donor animal. The skin thickness increased faster and reached a maximum earlier than when normal cells were injected. This enhanced reactivity of the blast cell population was seen only when the blasts were injected into the donor animal and did not occur when they

TABLE VII-4

Source of Cells	^{3}H-Thymidine		^{3}H-Uridine	
	Control cpm/10^6 cells	NLT cpm/10^6 cells	Control cpm/10^6 cells	NLT cpm/10^6 cells
Afferent Lymph	74	14,639	-	5,284
Efferent Lymph	271	2,607	288	899

The incorporation of ^{3}H-thymidine and ^{3}H-uridine into acid insoluble material by lymph cells coming from the site of an NLT lesion. Figures represent the mean of three samples. There were 10.1 per cent blast cells in the efferent lymph population and 20.1 per cent blasts in the afferent lymph cells.

FIGURE VII - 4



Dermal reactions produced in recipient sheep by normal efferent lymph cells and lymph cells containing 28% blasts obtained after the injection of homologous lymphocytes.

A. Reactions in the sheep which donated the homologous lymphocytes.

B., C. Reactions in two other sheep. The same number of cells was injected in each. Vertical bars represent the standard deviations of the mean of 3 skin reactions.

were injected into other recipients. In fact, in another experiment with a similar protocol, the reactions produced by the stimulated cells were again increased when injected into the specific donor but were reduced in comparison with the control lymphocytes when injected into other sheep. Efferent lymph cells containing blasts obtained during responses to influenza virus or Salmonella, produced lesions that were significantly smaller when compared with normal efferent cells (Figure VII-5).

It was concluded that cells containing a blast population were less reactive than normal lymphocytes in producing skin lesions except when the blasts that were collected during an NLT response were injected back into the donor sheep. In this latter situation, the specifically sensitized blast cell population was more reactive than normal lymphocytes.

The Ultrastructural Anatomy of an NLT Lesion

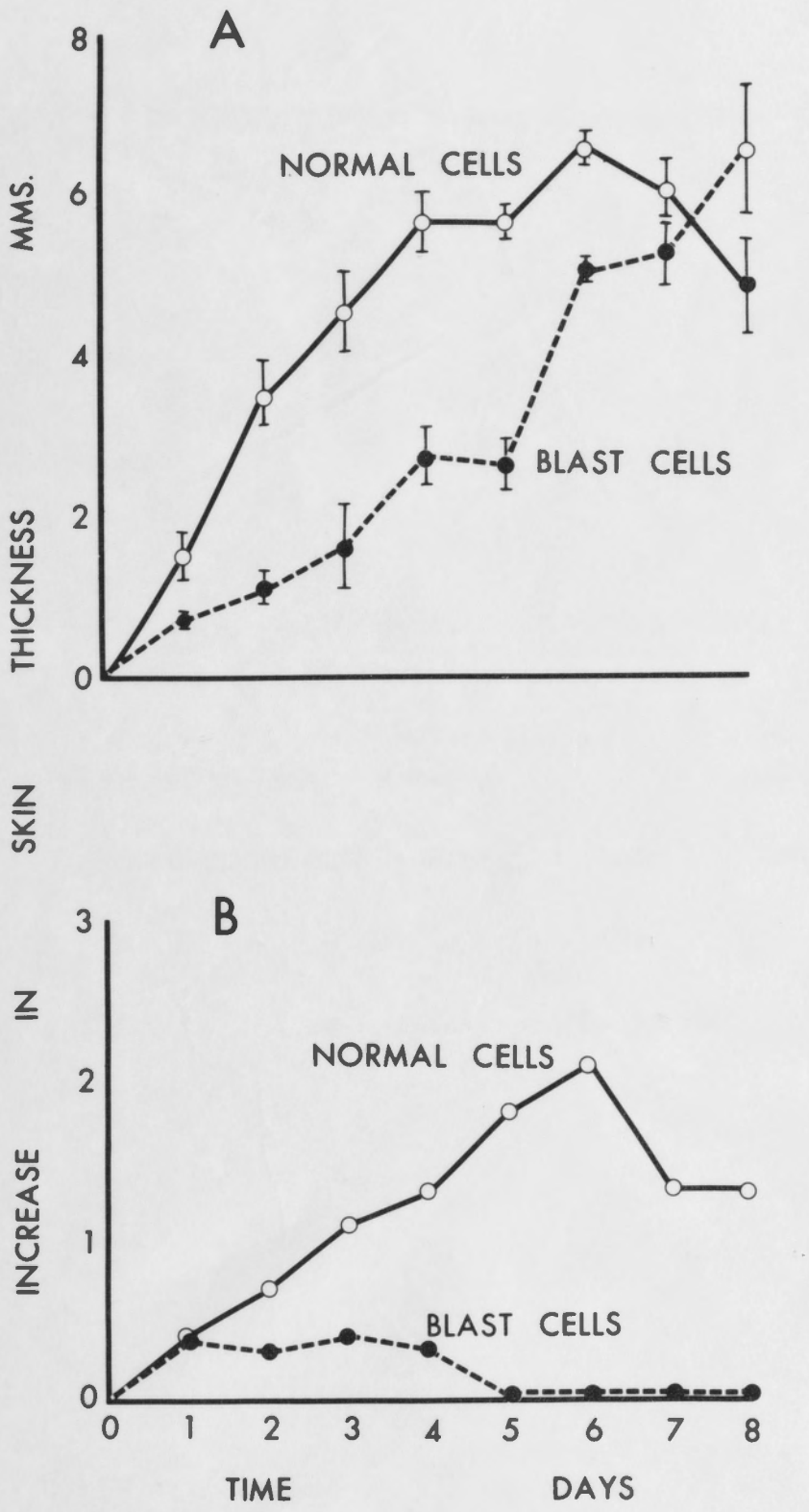
An NLT skin lesion was removed and fixed for electronmicroscopy 6 days after the lymphocytes had been injected. Ultrastructural changes were observed and compared with other homograft reactions in the sheep. The histology of the lesion is shown in Figures VII-6 to VII-9. The lesion was characteristically lymphoid in nature and contained very few polymorphonuclear leucocytes. Transformed lymphocytes, many of them in the process of division, were observed around blood vessels. An abundance of mature plasma cells was also seen but these cells were located in the connective tissues only. The endothelium of the blood capillaries and venules within the lesion contained many cytoplasmic organelles and lymphoid cells were observed closely associated with the endothelial cells inside the lumen of blood vessels.

Discussion

The NLT as a Graft-Versus-Host And a Homograft Reaction

Graft-versus-host reactions are homograft reactions in which an interaction of immunologically competent cells occurs with foreign homologous tissues. It is usually held

FIGURE VII - 5



Dermal reactions produced by the injection of normal, homologous efferent lymph cells and populations containing antigen-stimulated cells.

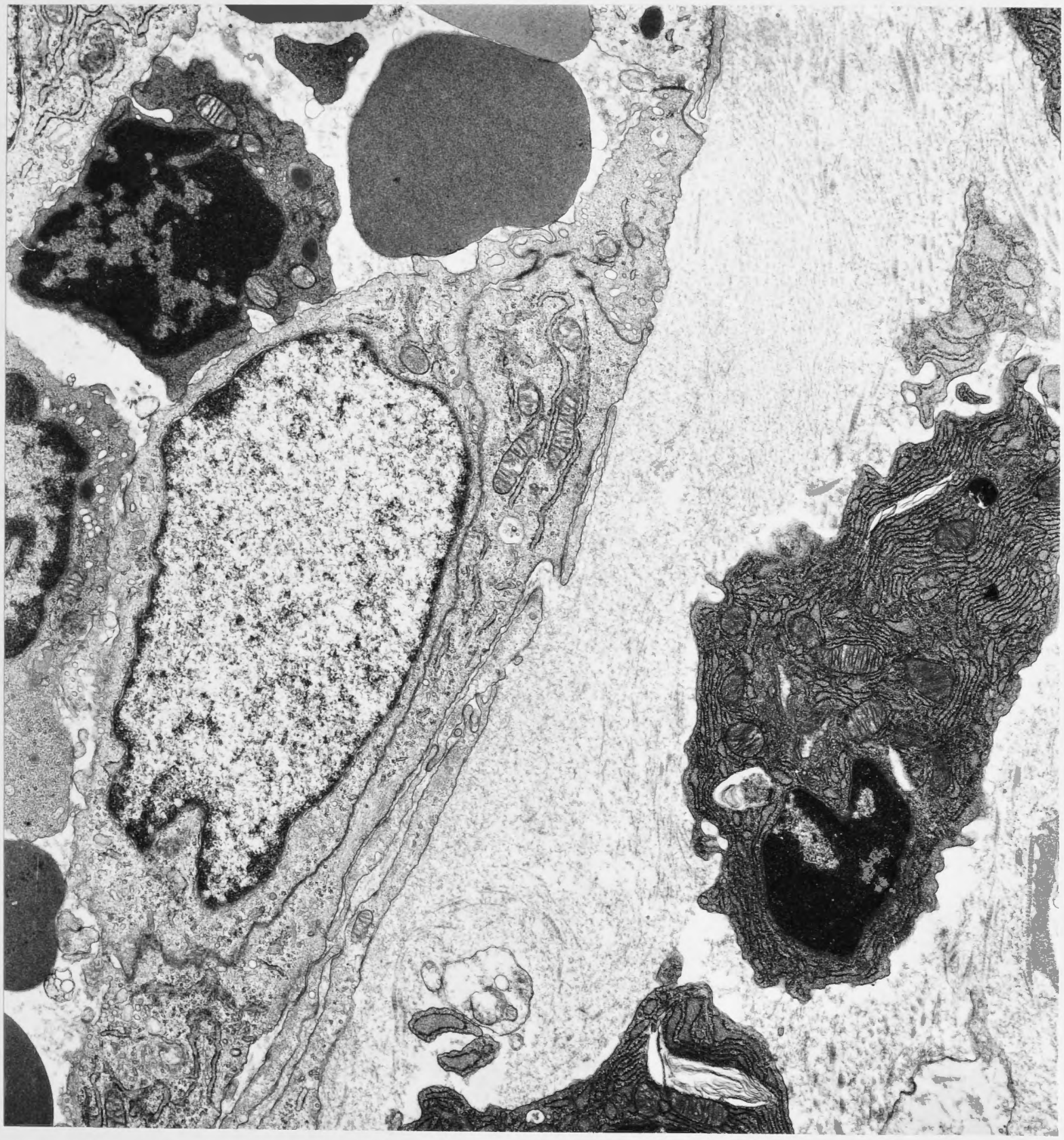
A. Population contained 51% blast cells obtained during an immune response to influenza virus.

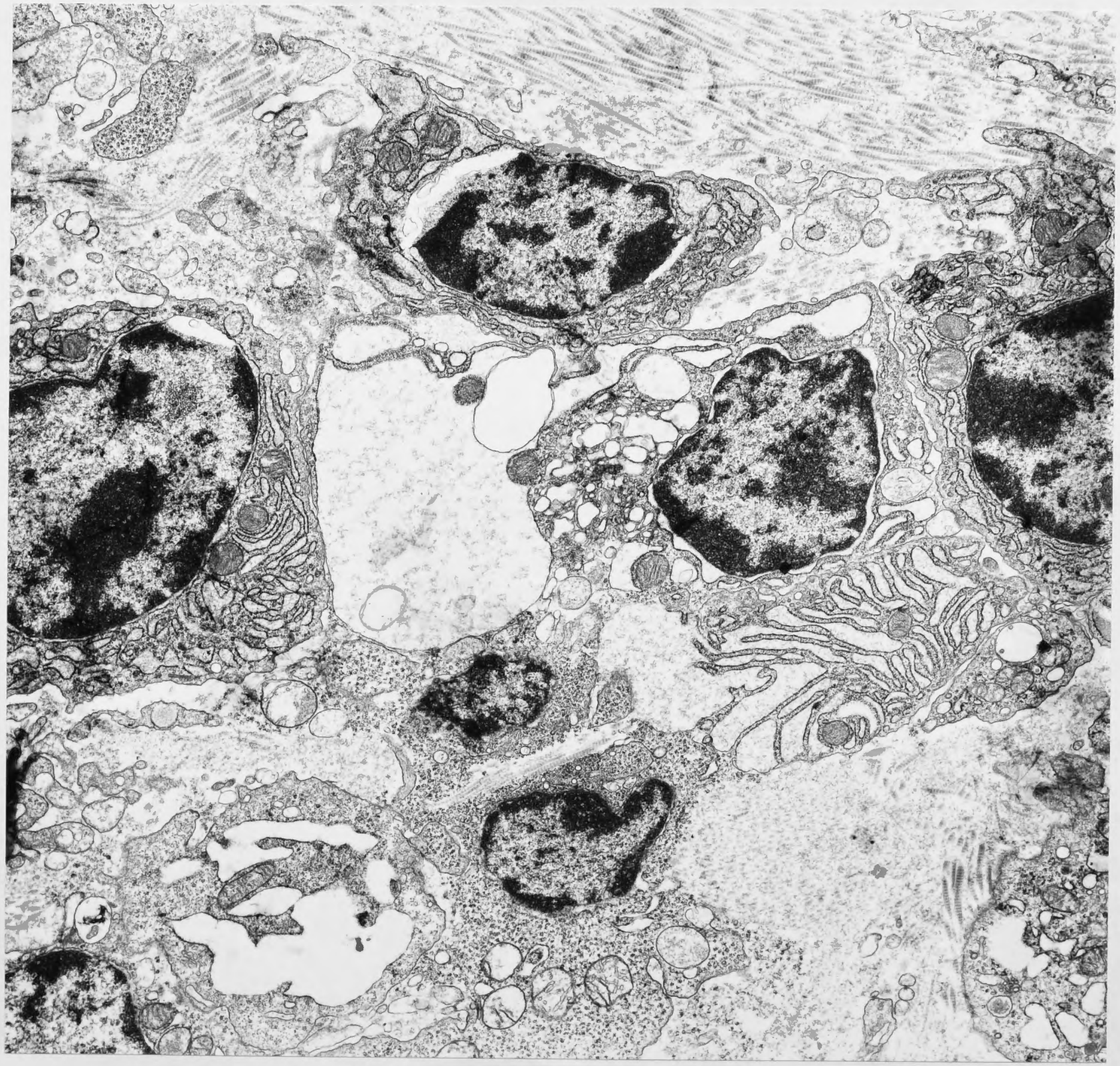
B. Population contained 88% blast cells obtained during an immune response to Salmonella. ALS was used in vivo to increase the proportion of blasts.

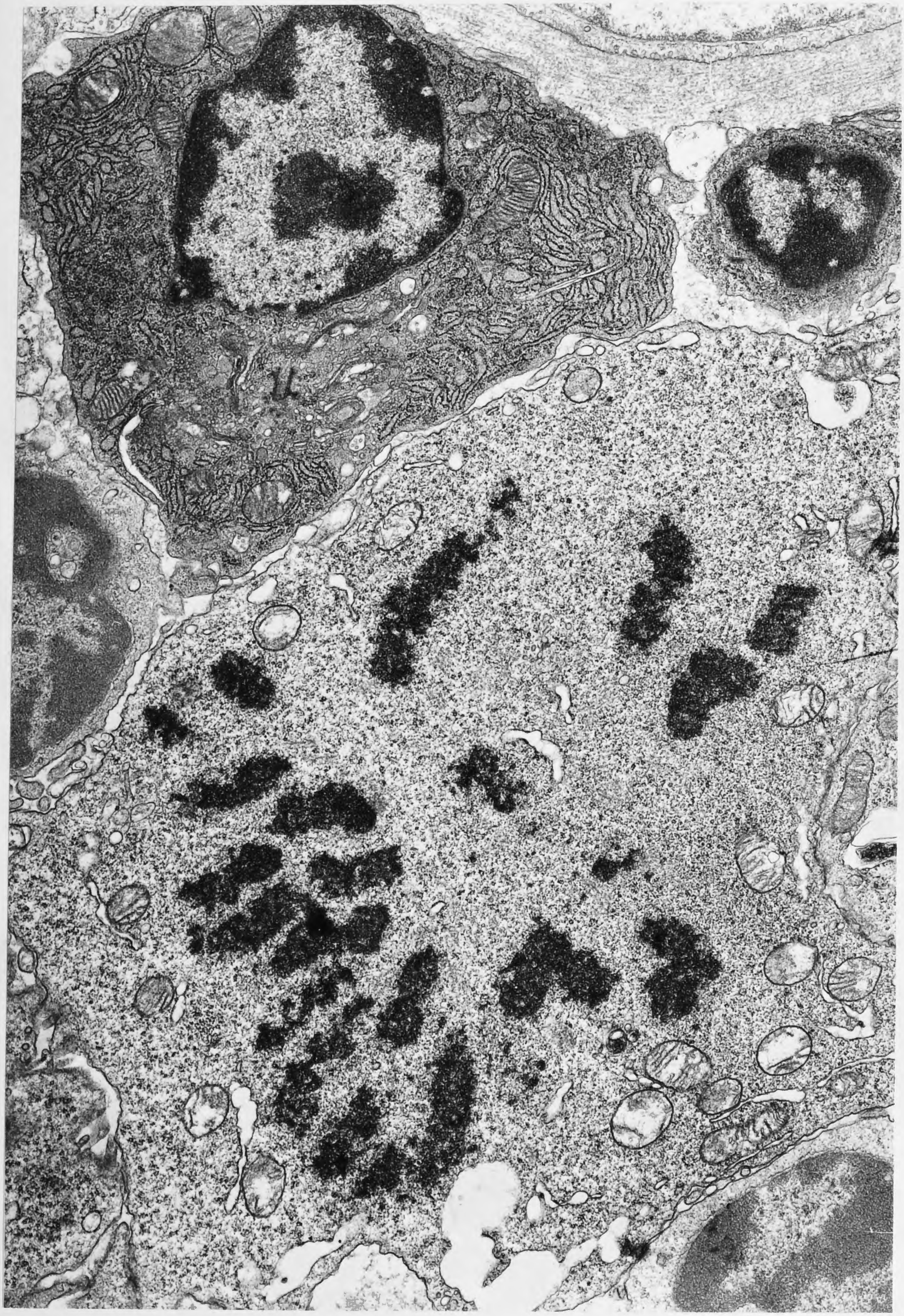
The same number of cells was injected in each. Vertical bars represent the standard deviations of the mean of 3 skin reactions.

FIGURES VII-6, VII-7, VII-8, VII-9

Electron micrographs of an NLT skin lesion 6 days after the injection of homologous lymphocytes. Note the well-developed cytoplasm of the capillary endothelial cells, the dividing lymphoid cells and the plasma cells. Magnifications X 1400.







that these reactions involve the reaction of lymphoid cells with foreign (histocompatibility) antigens (Gowans and McGregor, 1965). Subsequent pathological events in these reactions have been ascribed to the "aggressiveness" of immunologically competent cells.

The term graft-versus-host implies that the host is inert or at least has a limited potential to respond to the injected cells. The two situations where the host was previously considered unable to react were the chicken embryo graft-versus-host reactions and the reactions involving the introduction of parental cells from one isogenic strain into F_1 hybrid offspring of that strain and a second strain. Those reactions have since been shown to have some host component (Ramseier and Lindenmann, 1969 and Wilson, Schoefl and Lafferty, 1970).

There are however, examples of graft-versus-host reactions that have a considerable host component (Elkins and Guttmann, 1968; Ramseier and Streilin, 1965). The NLT reaction is one of these (Lafferty et al 1969). Mixed lymphocyte reactions consisting of cultures containing two homologous lymphocyte populations are regarded as two-directional reactions involving the stimulation of one group of lymphocytes by another and vice-versa. Regardless of whether the mechanism whereby lymphocytes are stimulated to respond in homologous situations differs from the stimulation of cells by antigen (Lafferty and Jones, 1969), homograft reactions in vivo produce extensive pathological changes in tissues.

Billingham, (1966) has summarized the pathology associated with different types of homologous reactions and Jones et al 1969) have described the pathology of the NLT skin reaction in the sheep as seen in the light microscope. The destructive nature of homograft reactions can be seen in several different circumstances. The function of homografts of whole organs such as kidneys and hearts are affected during these reactions. In other circumstances skin lesions, splenomegaly, runt disease, or death may occur. The involvement of both the afferent and efferent lymphatic

system in homograft reactions has been clearly demonstrated by Hall (1967) and Pedersen and Morris (1970).

The experiments described in this chapter have emphasized the tissue destruction associated with the NLT reaction and this can be observed macroscopically as a skin lesion, with the electron microscope and by the appearance in the lymph of increased levels of lysosomal enzymes as described in Chapter VIII. After a secondary challenge with donor lymphocytes, there was no NLT lesion formed but there was a conventional secondary immune response with the appearance of blast cells and specific antibody in the lymph.

Mechanisms of Tissue Damage in Homologous Cell or Tissue Reactions

a) Cell mediated mechanisms

Following the initial recognition process between lymphocytes and antigens or other lymphocytes, cell transformation and proliferation takes place. In the intact animal, some of the transformed cells produce and release antibody and some remain in the lymphoid system as "sensitized" cells. Most studies in transplantation biology have demonstrated or claimed to have demonstrated that one or other of the products of the immune response (specific cells or specific antibody) are the primary causative agents in the tissue destruction or graft rejection that is invariably observed. The transfer of reactivity by cells and not serum and similarities with delayed hypersensitivity reactions have favoured the interpretation that these reactions are cell-mediated (Medawar, 1958; Brent, 1958).

The function of sensitized or stimulated cells in these types of cell-mediated reactions is still not known. Various specific or non-specific products have been shown to come from stimulated lymphoid cells, or from the media of cultures containing them. Some of these products appear to have contradictory activities that are difficult to place in the context of tissue reactions. eg. macrophage migration inhibiting factor, chaemotactic factor for polymorphonuclear leucocytes, growth inhibiting factor, "transfer factor" in

the case of sensitized lymphocyte populations, and mitogenic factor (Smith and Good, 1968). In addition, there are many reports of an apparent direct cytotoxic action by sensitized, stimulated or normal lymphoid cells coming into close contact with nucleated target cells (Möller, 1965; Holm and Perlmann, 1965). Using sheep and collecting the efferent lymph cells produced during the immune response to mouse lymphoma cells, Denham, Hall, Wolf and Alexander, (1969) showed that the lymph cells destroyed target monolayers of mouse lymphoma cells. Möller, Zukoski, Lundgren, Beckman and Möller (1968) have shown that non-immune lymphoid cells are cytotoxic in vitro but they suggested that this process of allogeneic inhibition may not be an immunological phenomenon since granulocytes can also be cytotoxic to target cells.

In the experiments described in this chapter, it was evident that efferent lymph cells containing blasts produced in response to homologous lymphocytes were able to produce more severe reactions when injected subcutaneously into the donor sheep than did normal lymphocytes. Brent and Medawar (1963) and Ramseier and Billingham (1966) showed a similar type of enhancement by using sensitized cells for transfer reactions in guinea pigs and Syrian hamsters.

If tissue destruction in homologous reactions is mediated by effector cell-target cell interactions, a mixed lymphocyte culture should be a mutually self-destroying phenomenon. Instead, it is characterized by proliferation and lymphocyte differentiation. There is no evidence that cell destruction in these cultures is any higher than in control cultures or in lymphocyte cultures using PHA or other mitogens (Ling, 1968). In spite of numerous models for target cell destruction that have been examined in vitro, the only evidence for a similar phenomenon occuring in vivo is the suggested interaction between lymphoid cells and endothelial cells.

Several investigations have described reactive changes in vascular endothelial cells during homograft responses. Burwell (1962) studied vascular changes in lymph nodes

draining bone allografts in the rabbit and found that endothelial cells of postcapillary venules had an increased amount of intensely pyroninophilic cytoplasm and it was suggested that this was due to antigenic stimulation. "Reactive" changes in endothelium have also been described in sheep kidney homografts by Pedersen and Morris, (1970). They suggested that the interaction between the host lymphoid cells and the donor endothelium may be one of the crucial changes in the homograft. In the present study of the NLT lesion the endothelial cells also appeared reactive with polyribosomes and many cytoplasmic organelles similar to those described in the kidney homografts. Lymphoid cells in vessels were observed in close association with endothelial cells. In contrast to the kidney homograft situation however, the endothelial cells and the lymphoid cells within the vessels were almost certainly both of host origin and it seems unnecessary to postulate that any type of immunological interaction was taking place between these cells. It is possible that the changes in the endothelium are brought about by their modification to accommodate the increased cell traffic that is taking place from blood to lymph.

Postcapillary venules in the rat and other mammalian species normally are lined by endothelial cells which have abundant cytoplasm (Gowans and Knight, 1964). In contrast, endothelial cells of postcapillary venules in rats thymectomized at birth have little cytoplasm and the cytoplasm lacks its normal affinity for pyronin. Such rats are deficient in small lymphocytes and there is thus a smaller number of these cells recirculating through the postcapillary venules (Goldschneider and McGregor, 1968). Changes in endothelial cells may very well reflect the rate of movement of lymphocytes across the vessel wall and if this is so it is not necessary to invoke specific immunological phenomena. However, from the studies on systemic sensitization to kidney homografts (Strober and Gowans, 1965) it appears that foreign endothelium is probably capable of stimulating lymphocytes. There is, however, no direct evidence that

target cells are destroyed by lymphocytes or blast cells during homologous reactions in the animal.

b) Humoral mechanisms

Early investigations tended to neglect the role of antibody as the cause of tissue destruction in homologous reactions largely because iso-antibodies were not detectable. Recently, however, different assay systems have been devised and antibodies have been detected in many homograft situations (Iwasaki, Talmage and Starzl, 1967; Manzler, 1968; Spong, Feldman and Lee, 1968; Pedersen and Morris, 1970). Stetson (1963) and Morris (1969) have suggested that the importance of antibody in homograft rejection may be underestimated. Although specific antibody can be detected in homograft reactions it is not usually possible to determine its relevance or its mode of action in the intact animal.

While there is no doubt that antibody and antigen-antibody complexes may produce pathological changes in homograft situations (Kniker and Cochrane, 1968), these types of destructive reactions are not peculiar to homografts. The report of Cochrum, Davis, Kountz and Fundenberg, (1969), in which goat renal autografts were rejected after the passive transfer of immune plasma does suggest some role for antibody. In these experiments however, there was an extensive involvement of polymorphonuclear cells in the reaction and in this regard, the reaction seemed different from the pathology of clinical homograft rejection.

Many other agents are involved in the processes of homologous tissue damage or graft rejection such as platelet aggregation (Kountz, Williams, Williams, Kapros, and Dampster, 1963) Lowenhaupt and Nathan, 1968; fibrin (Guttmann, Lindquist, Parker, Carpenter and Merrill, 1967) histamine and serotonin (Mills, Robb and Roberts, 1968) and proteolytic enzymes (Porter, Dossetor, Marchioro, Peart, Rendall, Starzl and Terasaki, 1967). However, it is generally considered that the reactions in which these agents are involved are secondary to the primary immunological events (Shehadeh, Guttman, Lindquist and Rodriguez-Erdmann, 1970).

In some of the NLT responses antibody was detected in high titres by using the sensitive capillary tube migration assay (Thompson et al 1968). The role that this antibody plays in these situations, however, is not clear as it did not appear to possess any cytotoxic activity. This was in contrast to the cytotoxic nature of heterologous anti-lymphocyte antibody. This difference between the two antibodies may reflect a difference in their capacity to fix complement although this was not tested. Whilst it had no cytotoxic effect, the homologous antibody was capable of inhibiting the normal circulation of lymphocytes through the node and in this respect it appeared to be as efficient as heterologous ALS.

It was demonstrated by Jones et al (1969) that recipient sheep become refractory in terms of the NLT reaction to subsequent injections of homologous lymphocytes within a few days of the first injection and it was suggested that this was the result of an immune reaction of the recipient. Billingham (1966) studied NLT reactions in guinea pigs and he found that prior immunization of the prospective donor with recipient lymphocytes led to an enhanced capacity of the donor cells to produce a transfer lesion; similar results were described here using transforming cell populations containing blast cells reacting to donor lymphocytes. Prior immunization of the recipient however, resulted in no NLT reaction (Billingham 1966). This was similar to the results in sheep and it emphasizes that there must be a host-versus-graft component in both of these reactions. In the sheep a true secondary immune response against the grafted cells followed a secondary challenge with donor lymphocytes. If the NLT response is a two-way immunological reaction involving the stimulation of lymphocytes by hystocompatibility antigens, why does the secondary response not result in tissue damage (NLT lesion) similar to or greater than the primary response? Not only was there no reaction at the injection site but the failure to find an increase in β-glucuronidase levels in efferent lymph suggested that there was no tissue damage produced in the lymph node either. A secondary NLT response

thus does not result in a proliferative, destructive reaction, even though the histocompatibility antigens of the injected cells still initiate a secondary immune response. In terms of current immunological dogma, this dilemma cannot be rationalized. Lafferty (1970) suggested that the graft-versus-host reaction is a pseudo-immunological event which involves cellular recognition and stimulation between immunologically competent, homologous cells possibly mediated by an RNA transfer (Jones and Lafferty, 1968) rather than by histocompatibility antigens. Such an explanation however still begs the question of how the extensive tissue damage occurs in these reactions.

There is a possibility that the mechanism of primary tissue destruction in homologous reactions is independent of the direct action of normal or sensitized cells on target tissue and that the production of antibody in these reactions is also irrelevant. The following propositions are concerned with an alternative explanation.

c) <u>An alternative hypothesis</u>

A rejecting kidney homograft (Pedersen and Morris, 1970), a chronic granuloma (Smith, McIntosh and Morris, 1970) and NLT lesions show some striking similarities to a lymph node reacting to an antigen. Lymphocytes, proliferating blasts and plasma cells are prominent cell types in all these situations. In addition, cell circulation through these tissues is enhanced and the rate of lymph flow is also increased. There appears to be no doubt that a local immune response is occurring in each situation although the structure of the tissues where this is taking place varies considerably.

A lymph node is an organized, structured, aggregate of lymphoid and supportive tissue elements. There are specialized vessels, the post-capillary venules, to facilitate high rates of lymphocyte circulation. A rapidly proliferating immune response initiated in the cortex (probably in a germinal centre) can and does expand into well-developed lymph sinuses throughout the medulla and from these sites cells can readily escape and overflow into the efferent lymphatics. Fixed phagocytic cells are situated

along the lymph sinuses to assist in removing and catabolizing excess antigen. During an immune response a lymph node weighing 1 gram may export its own weight of stimulated cells in the efferent lymph and in this time, the node itself may be traversed by as much as 5 grams of lymphocytes circulating from the blood to the efferent lymph. The flow of lymph through the node can increase up to five times and when the immune response has receded, the node may still weigh 2-4 times its initial weight.

Throughout all of this the node continues to function, in fact it may perform its function more efficiently after another, secondary challenge although prolonged antigenic challenge usually leads to fibrosis of the node.

It is possible that other types of specialized tissue like kidney, muscle or subcutaneous tissue might have difficulty maintaining their functional and structural integrity when an immune response involving an extensive infiltration and proliferation of cells is taking place. Instead of having an organized structure to process foreign material delivered to it in special absorbing vessels as in the case of a lymph node, other types of tissues must recruit the necessary lymphoid and phagocytic cells from the circulation. That is, rather than having the antigen delivered to the lymphoid tissue, the lymphoid tissue must be delivered to the antigen. In the case of a grafted organ, there is a very large quantity of this antigen. It is difficult therefore to imagine how any specialized tissue structure could increase its mass by a factor of 2-4 times with "foreign" cell types, develop and extend specialized lymphatic and circulatory pathways to handle the increased traffic of cells from blood through the tissue to the lymph, and at the same time accommodate a population of rapidly proliferating lymphoid cells without suffering some degree of damage. The primary mechanism of tissue destruction in homologous reactions may be the direct result of these aspects of an immune response in non-lymphoid tissues.

Experiments can be designed to test this proposition. However, there is already some evidence to support it.

Examination of the electron micrographs from an NLT lesion shows large numbers of plasma cells in the connective tissue of the skin. In addition, large blasts, some in mitosis, are commonly seen. It is not difficult to imagine these cells interfering with the normal functioning of the collagen and connective tissues with which they are associated in exactly the same manner as infiltrating polymorphonuclear cells or neoplastic cells interfere with normal structure and function in conditions of acute inflammation or malignancy by displacing, primarily in a physical manner, differentiated tissues which are often made up of functional units (eg. nephrons, thyroid follicles) of cell aggregates.

Such an hypothesis does not explain the features of homologous reactions which are related to the inductive mechanisms involved in cell recognition and transformation. However, the inductive processes of lymphocyte stimulation are likely to be similar whether the immune response is transpiring in fixed lymphoid tissue or in a non-lymphoid tissue.

The reasons why homologous lymphocytes in an NLT reaction lead to the formation of a lesion at the injection site rather than in the regional lymph node as occurs with heterologous cells, remains unanswered. Similarly, why are homologous lymphocytes in a secondary response treated in a manner similar to heterologous cells? Antibody may play a role in this phenomenon but if it does so, the manner in which it acts is unknown.

In summary it is suggested that tissue destruction in homologous reactions is primarily a manifestation of a normal immune response which takes place in tissues that are not specialized to accommodate this type of cellular reaction. Fixed lymphoid tissue on the other hand is specifically organized to cope with the migratory, proliferative, and infiltrative aspects of an immune response and can do so without compromising its physiological activities although even in this specialized organ an immune response has certain consequences which lead to a degree of lymph node pathology. Graft destruction in terms of the specific action of

cellular or humoral products of a particular immune response may in fact be an incidental phenomenon.

Summary

1. The changes that occurred in lymph draining from an NLT lesion revealed the large contribution of host cells to this reaction. There was an immune response at the injection site as well as in the draining lymph node. The dynamics of the cell response and lymph flow during an NLT response were similar to other homograft situations.

2. The ultrastructural anatomy of an NLT lesion also showed similarities to other homograft situations including the presence of "reactive" endothelium. No specific immunological significance was attached to this in terms of lymphoid cell-target cell interaction because the lymphoid cells and the endothelium were almost certainly both of host origin.

3. The cells draining from an NLT reaction were capable of specifically enhancing a skin reaction when injected back into the donor animal. In contrast other antigen-stimulated cells were less reactive than normal lymphocytes.

4. During a primary response to homologous lymphocytes, there was extensive tissue damage with the production of a local NLT lesion as well as antibody production. After a secondary challenge, however, no lesion developed and there was no evidence of tissue damage. In these circumstances the immune response in the host had the characteristics of a secondary immune response with the production of specific anti-lymphocyte antibody.

5. No cytotoxic activity or lymphocyte transforming capacity was associated with homologous anti-lymphocyte antibody although this antibody was capable of modifying the normal circulation of lymphocytes in the donor sheep.

6. Mechanisms of tissue destruction in homologous reactions based on cell-mediated immunity and humoral antibody do not adequately explain the observed phenomena and an alternative hypothesis is discussed.

CHAPTER VIII

LYSOSOMAL ENZYMES IN LYMPH AND THEIR RELATIONSHIP TO IMMUNOLOGICAL EVENTS

Lysosomal Enzymes in Lymph and Their Relationship to Immunological Events

Although changes in lysosomal enzymes occur in a variety of pathological and physiological processes, (de Duve, Pressman, Gianetto, Wattiaux and Applemans, 1955; Woessner, 1969; Dott, 1969; and Vaes, 1969) no specific role has been ascribed to them in the primary immune response (Bowers, 1969). However, in view of the scarcity of information available on the subject this possibility is still open. Lysosomes have been identified histochemically in lymphoid tissue where they occur in large numbers in phagocytic cells. Barka, Schaffner, and Popper, (1961) demonstrated the localization of acid phosphatase, particularly in the reticular cells and in the endothelial cells of lymph sinuses, in reticular cells of lymphoid follicles and in phagocytic cells in the follicles of rat lymph nodes. The localization of N-acetyl-β-glucosaminidase in macrophages and reticular cells of rat lymph nodes has been described by Hayashi, (1964, 1967). Compared with phagocytic cells, lymphocytes appear to be poorly endowed with lysosomes (Braunstein, Freiman, Thomas and Gall, 1962) but they do apparently contain particles which stain positively for acid-phosphatase (Allison and Mallucci, 1964; Diengdoh and Turk, 1965; Tanaka and Liddy, 1966) and the number of these particles appears to increase after stimulation with phytohaemagglutinin (Hirshhorn, Kaplan, Goldberg, Hirshhorn and Weissman, 1965; Parker, Wakasa and Lukes, 1965), or following skin sensitization with oxazolone (Diengdoh and Turk, 1965).

Most biochemical studies on the lysosomal enzymes in lymphoid tissue have been done with extracts from whole spleens or lymph nodes. The experimental approach has been to expose animals to X-irradiation or treat them with steroids so as to reduce the content of lymphocytes in these tissues and provide a base level of enzyme activity. The enzyme content of lymphocytes was then assessed indirectly by comparing the activity in these tissues before treatment and after they have been depleted of these cells (Bowers and de Duve, 1967; Bowers, 1969).

The experiments reported in this chapter were designed to examine the changes in some of the lysosomal enzymes in the free-floating cells of lymph, particularly acid-phosphatase, β-glucuronidase and N-acetyl-glucosaminidase, before and following antigenic stimulation. The levels of these enzymes in the lymph plasma were also determined.

Results

It became apparent at an early stage of this investigation that there were significant changes in the levels of enzymes both in the lymph plasma and in the lymph cells during the immune response. However, the immune response as it occurs in the intact animal involves a variety of interacting phenomena quite apart from those directly related to the synthesis of specific antibody and these phenomena may in themselves produce changes in the enzyme activity. In some cases a transitory inflammatory episode is superimposed on the immunological events, surgical trauma invariably involves some degree of tissue damage as does the injection of antigen into the lower leg. In addition, some antigens may contain pyrogens or other materials which produce systemic effects in the animal. The involvement of cells other than lymphocytes and alterations in blood capillary permeability must also be taken into account when assessing the significance of changes occurring in the lymph. Because of this, experiments were first designed to investigate the alterations in enzyme activity that are associated with inflammatory responses and tissue damage so as to give a proper perspective to the immune response.

Changes in Enzyme Activity in Lymph and in Lymph Cells During the Inflammatory Response to Helminth Parasite Antigens

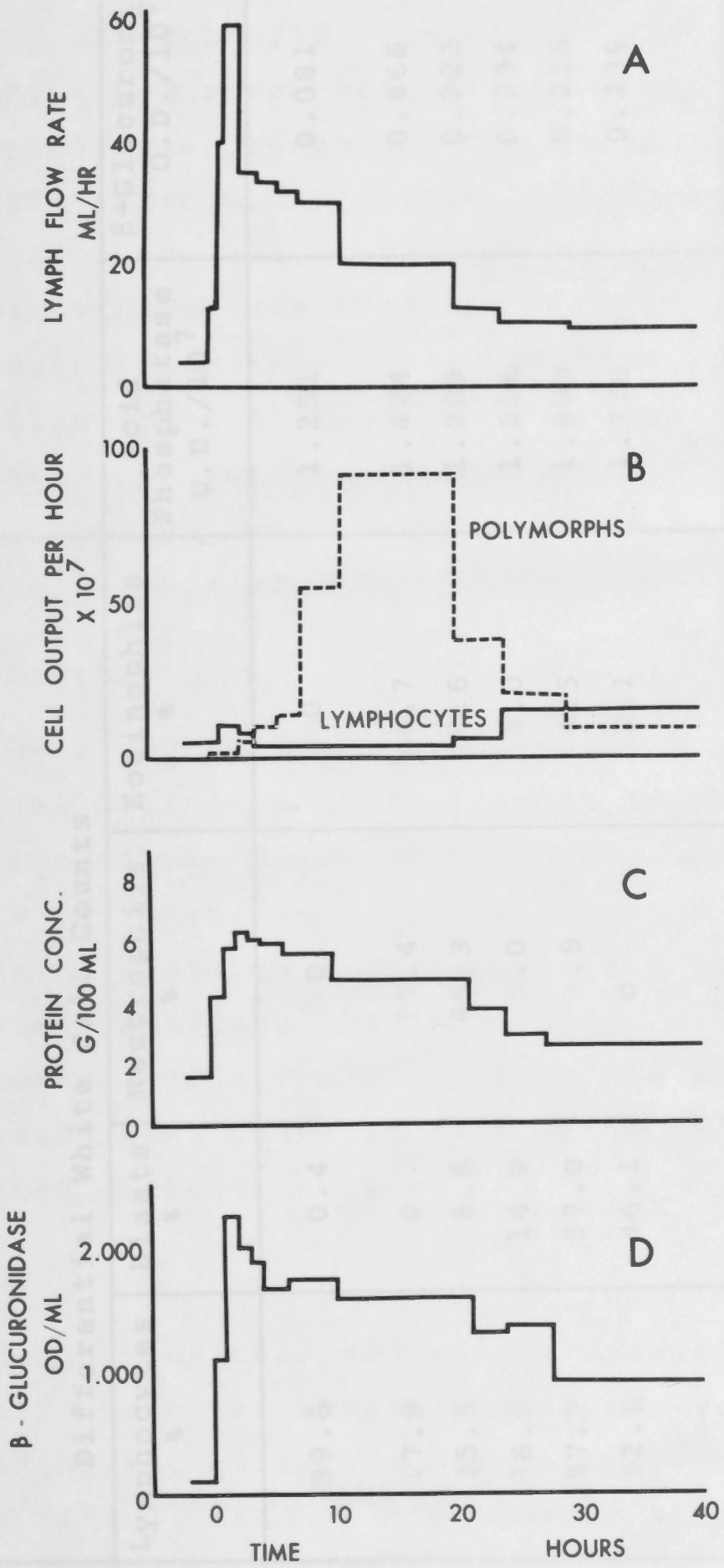
In order to study enzyme changes in the inflammatory response a solution of Echinococcus antigens was injected into the lower leg of a sheep and the efferent popliteal

lymph was collected. As was described in Chapter VI this material produces an extremely severe polymorphonuclear cell reaction together with drastic alterations in capillary permeability.

The results of this experiment are described in Figure VIII-1. Within the first hour after injection the flow rate increased and polymorphonuclear cells appeared in the lymph. Simultaneously there was a significant increase in the protein and β-glucuronidase content of the lymph plasma.

The precise origin of the β-glucuronidase was difficult to determine. The protein content of blood plasma is normally about 3 times the protein content of efferent lymph. However,this distribution does not appear to be the same for β-glucuronidase, the blood:lymph ratio for this enzyme being 10-20 to 1. During the first three hours of this inflammatory response the lymph protein concentration increased by a factor of 3 and reached approximately the level of the blood plasma. The β-glucuronidase activity also approached the blood plasma levels and suggested that this was the source of the enzyme. During this period the lymph flow rate reached 59 ml/hr. Several hours later the maximum output of polymorphonuclear cells occurred. While there was a significant decrease in the plasma protein concentration of the lymph at this time the level of β-glucuronidase in the lymph remained high, suggesting that at this stage of the response the β-glucuronidase was being released from cells associated with the inflammatory response. Table VIII-1 shows the types of cells found in the lymph and the activity of the lysosomal enzymes in the mixed cell population. In a population containing 82.1 per cent polymorphonuclear cells the acid-phosphatase activity was only 20 per cent higher than the activity found in a population of pure lymphocytes. In contrast, the activity of β-glucuronidase in the predominantly polymorphonuclear population was approximately 5 times the lymphocyte value. In another similar experiment (experiment 2, Table VIII-1)

FIGURE VIII - 1



An inflammatory response to Echinococcus antigens as measured in the efferent popliteal lymph.

TABLE VIII-1

EXPERIMENT 1	Differential White Cell Counts						
Time after Antigen hr	Lymphocytes %	Blasts %	Neutrophils %	Eosinophils %	Acid Phosphatase O.D./10^7	β-Glucuronidase O.D./10^7	Units LDH
BEFORE INJECTION	99.6	0.4	0	0	1.251	0.081	
24-27	17.9	0	77.4	4.7	1.474	0.466	
27-45.5	35.5	6.6	49.3	8.6	1.009	0.223	
45.5-50	76.0	14.0	5.0	5.0	1.516	0.234	
50-74	57.7	37.0	0.8	4.5	1.909	0.255	
74-74.5	52.8	46.1	0	1.1	1.778	0.239	
EXPERIMENT 2							
BEFORE INJECTION	99.8	0.2	0	0	0.771	-	275
6-10	11.0	1.3	68.2	19.5	2.020	2.032	500
10-24	31.4	2.3	46.1	20.2	2.234	1.659	337

The enzyme levels in lymph cells collected after the injection of Echinococcus antigens.

the activity of lysosomal enzymes as measured by the acid-phosphatase and β-glucuronidase activity showed similar changes, the acid phosphatase activity of the predominantly polymorphonuclear population being about 3 times the value found in a lymphocyte population and the β-glucuronidase activity being increased 20 fold. The percentage of eosinophils was greater in the second experiment than in the first and could possibly have accounted for the increased levels of these enzymes in the second experiment but no further evidence was obtained to substantiate this association. As the release of some of the β-glucuronidase from polymorphonuclear cells could have accounted for a significant proportion of the lymph plasma levels of this enzyme, the following experiments were carried out to further assess this possibility.

EXPERIMENTAL

A cell population containing 66 per cent polymorphonuclear cells was collected from efferent lymph 10-24 hours after the injection of the parasite antigens. Samples of these cells (4.28×10^7) were centrifgued from the lymph, washed once with Eagle's medium and re-suspended in 2 ml of either lymph or Eagle's medium containing 20 per cent lymph. Antigen solution (0.025 ml) was added to the sample tubes and incubated for 2 hours at $37^{\circ}C$ while control tubes were incubated without antigen.

RESULTS

When the culture media were assayed for β-glucuronidase activity there was about 5 times more activity in the samples containing the cells incubated in lymph plus antigen (Table VIII-2) than in the control samples. When the cells themselves were examined for their residual β-glucuronidase content at the end of 2 hours those samples incubated in the presence of antigen showed a significant decrease in enzyme content while those incubated in the absence of antigen did not.

TABLE VIII-2

Incubation Medium	Activity in Medium O.D./ml	Residual Activity in Cells O.D./10^7
Lymph + Echinococcus antigen	0.806	0.644
Eagle's containing 20% lymph + Echinococcus antigen	0.190	1.011
Eagle's containing 20% lymph	0.154	1.312

The release of β-glucuronidase from polymorphonuclear cells <u>in vitro</u>. The optical density values expressed represent the mean of duplicate cultures after blanks containing medium only had been subtracted.

Further experiments showed that acid phosphatase was also released under the same conditions. However, when antigen was added to lymphocytes no release of β-glucuronidase was observed. Further support for the release of enzymes from polymorphonuclear cells was obtained by incubating cells collected during the inflammatory phase of a secondary response to horse-radish peroxidase in the presence of lymph plasma and antigen. In this experiment similar results were obtained.

It can be concluded from these experiments that the increased level of β-glucuronidase detected in the lymph plasma during the inflammatory response arose, in part, from the large numbers of neutrophils and eosinophils associated with the reaction. The enzymes detected were released only in the presence of lymph and antigen.

The Effect of Tissue Damage Within a Lymph Node on the Levels of Lysosomal Enzymes Appearing in the Lymph

These experiments were designed to produce tissue and cellular damage within a lymph node by using the cytotoxic drug Vinblastin sulphate (Lilly). It was argued that in these circumstances the changes observed in the levels of enzymes in the efferent lymph would be due to the destructive action of the drug on lymphoid cells without any concomitant immunological or inflammatory reaction within the node.

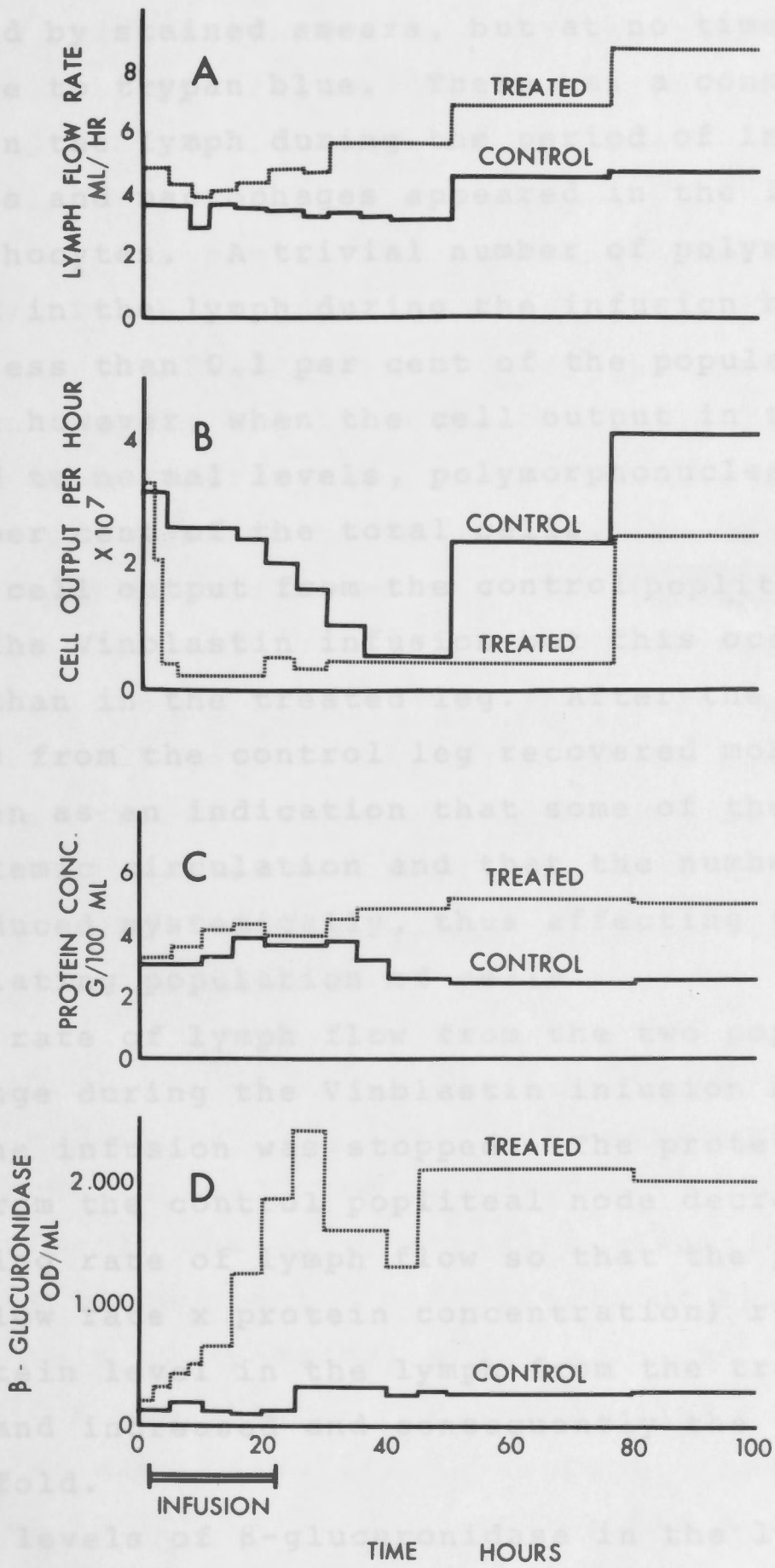
EXPERIMENTAL

Vinblastin was infused into the left popliteal node of a sheep via an afferent lymphatic at a rate of 0.5 ml per hour for 22 hours. The total amount of the drug administered was 5 mg. Both the left and right efferent popliteal lymphatics were cannulated and the right lymphatic served as a control for measuring the systemic effects of the drug.

RESULTS

The details of this experiment are shown in Figure VII-2. The cell output of the node was reduced to 10-20 per cent of normal during the infusion of Vinblastin and remained at this

FIGURE VIII - 2



The effect of a continuous infusion of Vinblastin on the efferent popliteal lymph.

level for 24-48 hours after the infusion was stopped. During this time the types of cells in the lymph did not change appreciably, over 98 per cent being small lymphocytes. Some of the lymphocytes appeared to be damaged by the Vinblastin as judged by stained smears, but at no time were they permeable to trypan blue. There was a considerable amount of debris in the lymph during the period of infusion and a few monocytes and macrophages appeared in the lymph together with the lymphocytes. A trivial number of polymorphonuclear cells appeared in the lymph during the infusion but these were always less than 0.1 per cent of the population. After the infusion however, when the cell output in the lymph had returned to normal levels, polymorphonuclear cells accounted for 10 per cent of the total cells.

The cell output from the control popliteal node also fell during the Vinblastin infusion but this occurred much more slowly than in the treated leg. After the infusion the output of cells from the control leg recovered more quickly. This was taken as an indication that some of the Vinblastin entered the systemic circulation and that the number of lymphocytes were reduced systemically, thus affecting the total recirculating population of cells.

The rate of lymph flow from the two popliteal nodes did not change during the Vinblastin infusion but it increased after the infusion was stopped. The protein content of the lymph from the control popliteal node decreased with the increasing rate of lymph flow so that the protein output per hour (flow rate x protein concentration) remained constant; the protein level in the lymph from the treated node on the other hand increased and consequently the protein output rose 4 fold.

The levels of β-glucuronidase in the lymph from the two legs were very different. The amount of activity in the lymph from the Vinblastin treated node increased during the period of infusion to reach a level more than 10 times the preinfusion level and more than 10 times the level in the control leg.

Contrary to the response to the parasite antigens this increase in the β-glucuronidase activity was not accompanied by any appreciable change in flow rate, protein content, or polymorphonuclear cell content of the lymph. It was concluded therefore, that most of the enzyme that appeared during this period resulted from the destruction of the tissue and cells within the lymph node.

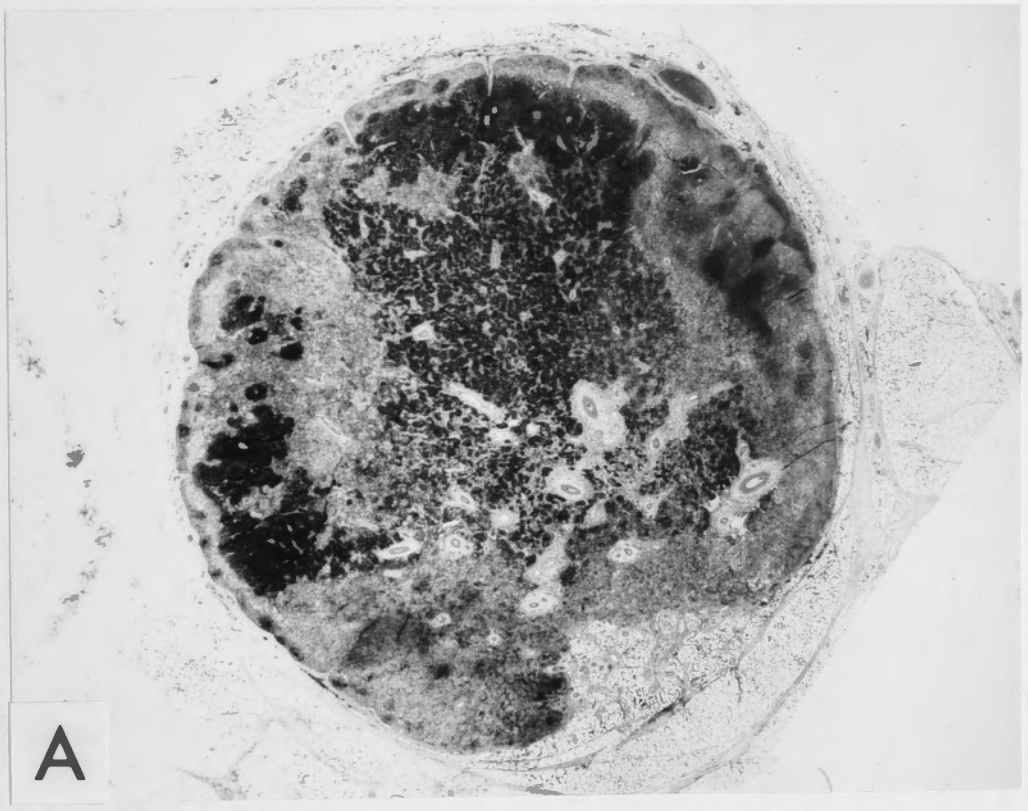
The popliteal nodes were removed at 96 hours after the start of the Vinblastin infusion and examined histologically. There was tissue destruction only in the treated node, the damage being confined primarily to the cortex with destruction of the follicular architecture (Figure VIII-3 and VIII-4). Cells in the medullary region of both the treated and the control node appeared to be unaffected. It was concluded that Vinblastin exerted most of its action on the tissue closest to the pericapsular sinus where the afferent lymphatic entered the node. Some of the drug must have gained access to the circulation directly through the blood capillaries of the node thereby producing the systemic effect that was observed. It seemed probable that very little of the Vinblastin actually reached the efferent lymph, or for that matter, even the medulla of the node.

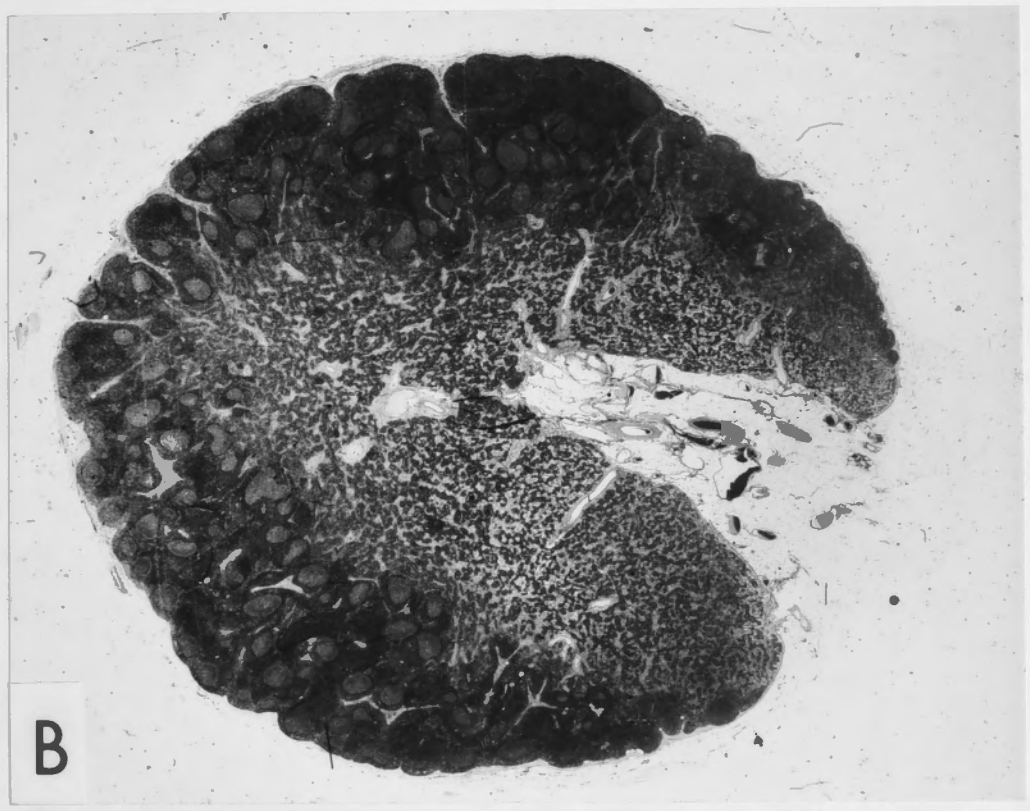
The Levels of Enzyme Activity in Lymph and in Lymph Cells During the Response to Homologous Lymphocytes

The results described in Chapter VII showed that the NLT reaction in the sheep contains a substantial host-versus-graft component. The effects of this type of reaction on the levels of lysosomal enzymes in the lymph were examined for two reasons. Firstly, this reaction displays all the features of an immune response, and as such is considered in the following section and secondly, local tissue destruction is a prominent feature of the reaction.

Figure VIII-5 shows the activity of β-glucuronidase that was measured in the lymph plasma during both the primary and the secondary responses to homologous lymphocytes. The enzyme level in efferent lymph collected during the

FIGURE VIII - 3

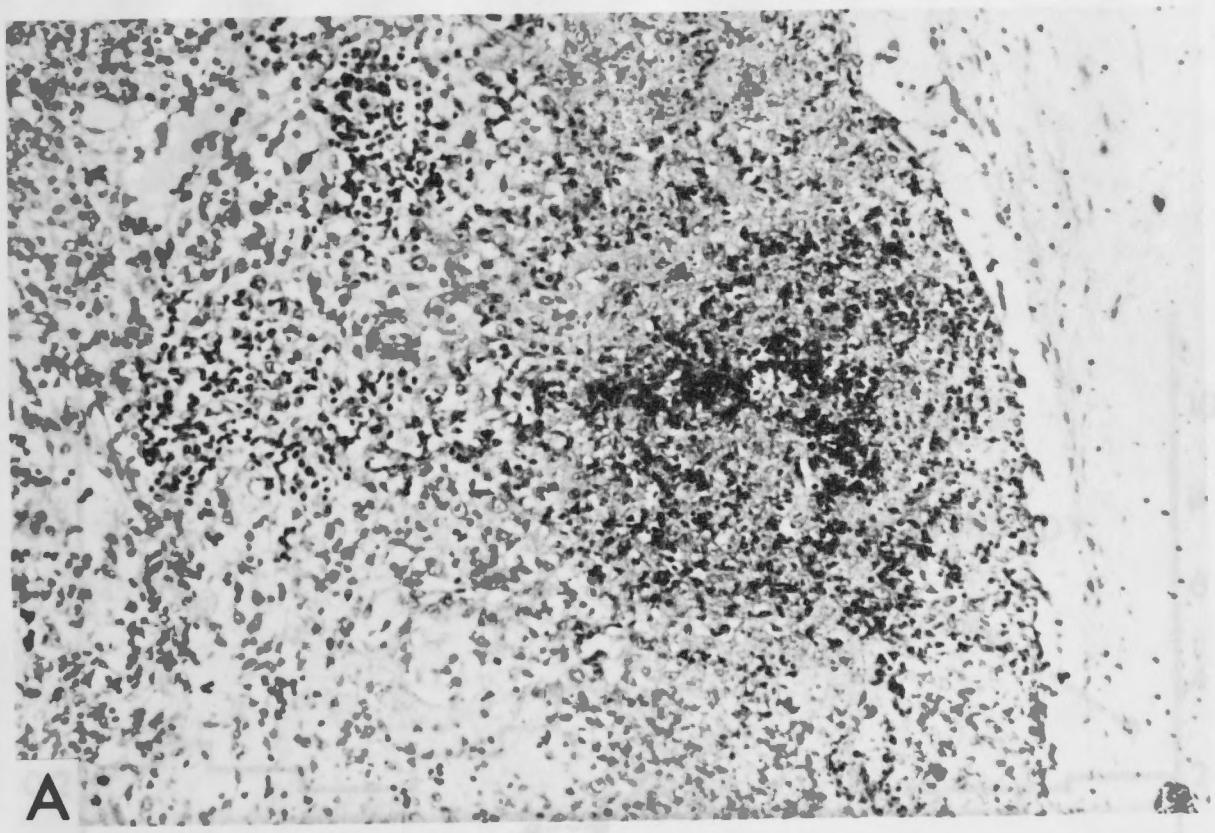


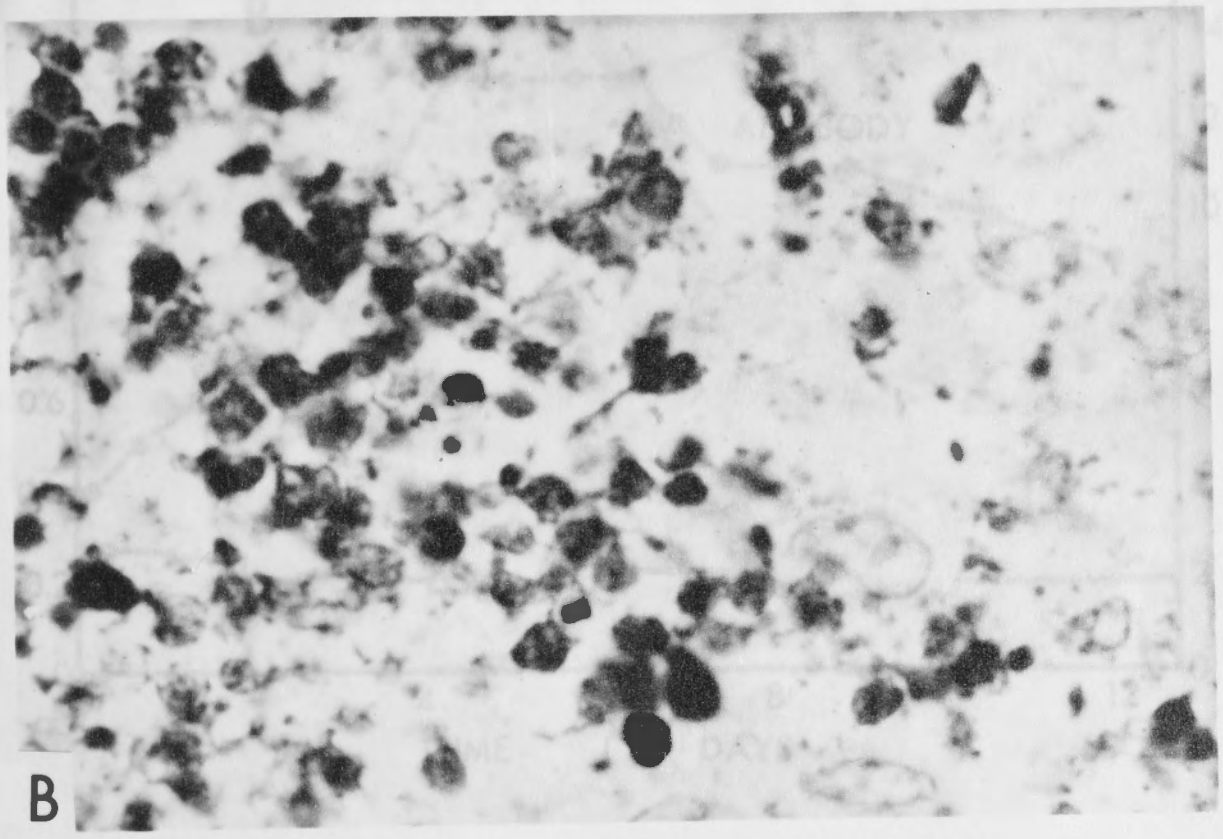


A. The histological appearance of a popliteal lymph node removed 4 days after the infusion of 5 mg. of Vinblastin via an afferent lymphatic.

B. The histological appearance of the control popliteal lymph node from the same sheep.
Stain - Haematoxylin and eosin.
Magnification x 5.

FIGURE VIII - 4



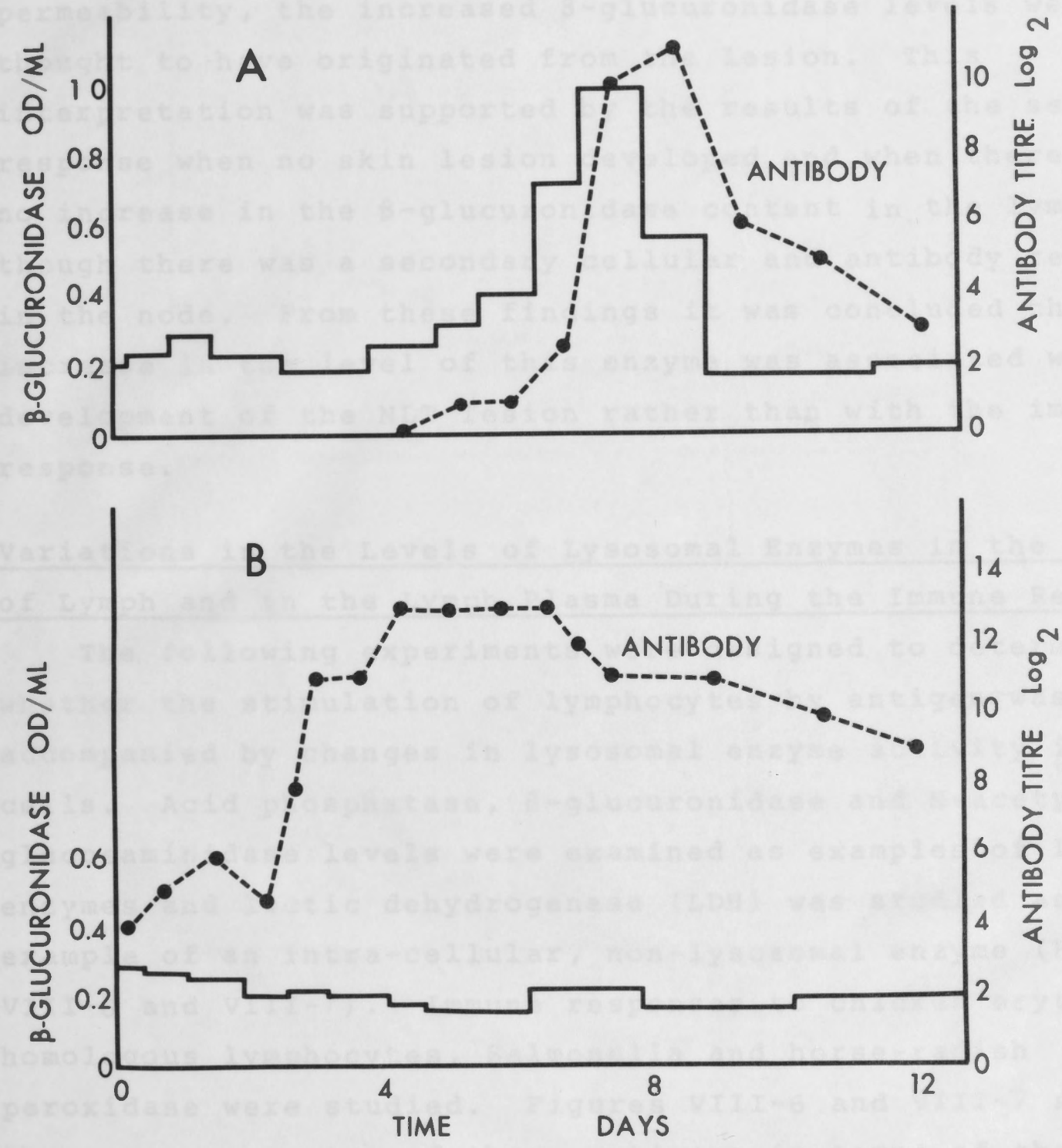


Extensive necrosis of a lymph node follicle after the infusion of vinblastin. Haematoxylin and eosin stain.

A. Magnification x 160

B. Magnification x 700.

FIGURE VIII - 5



The levels of β - glucuronidase and antibody in efferent lymph during a primary (A) and a secondary (B) response to homologous lymphocytes.

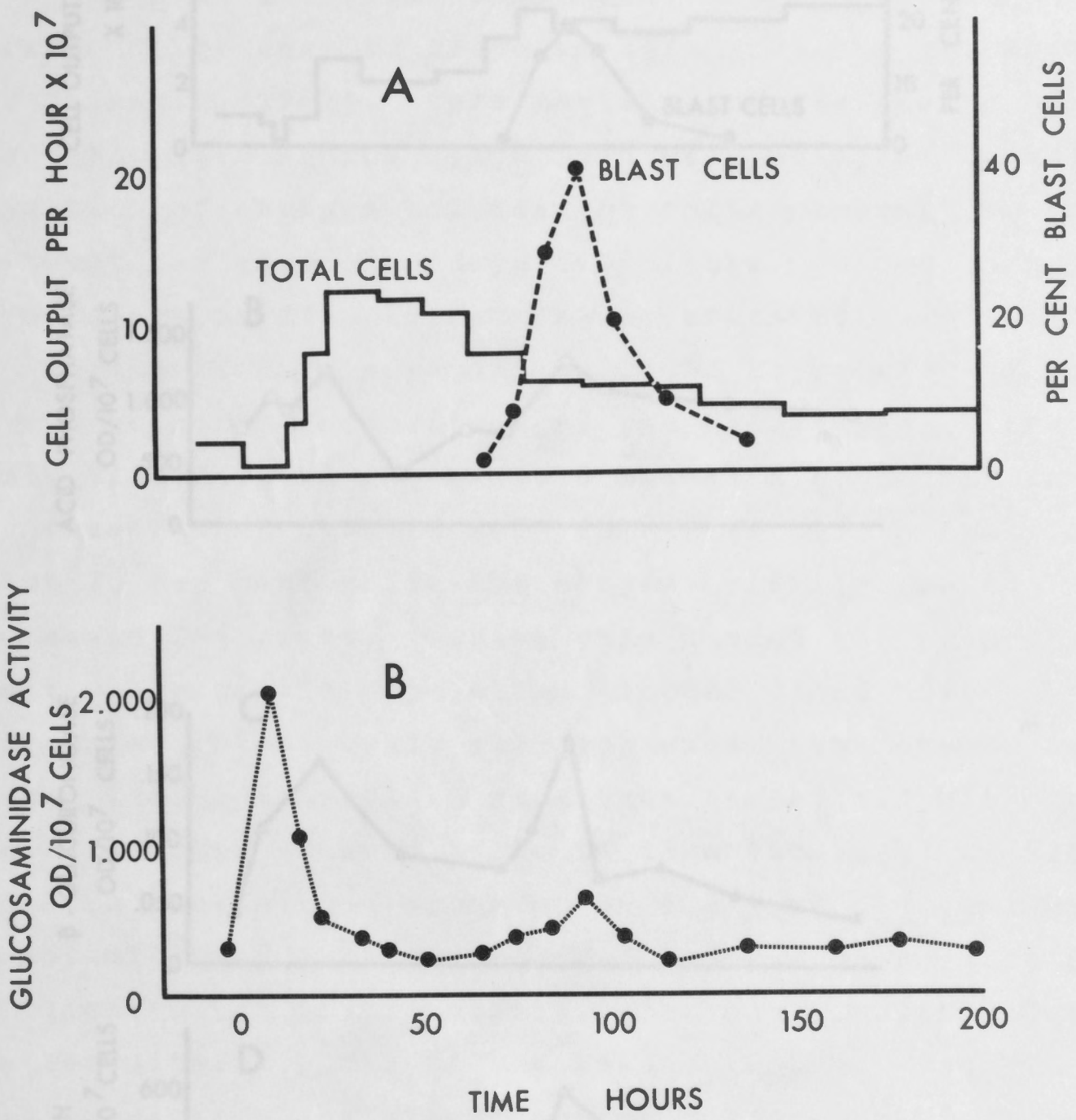
primary response increased as the blast cells appeared and at the height of the response, the levels of β-glucuronidase activity were increased 5 fold. The NLT skin lesion was near its maximum size at this time. Since there was very little change in the protein content or flow rate of the lymph which would have indicated alterations in capillary permeability, the increased β-glucuronidase levels were thought to have originated from the lesion. This interpretation was supported by the results of the secondary response when no skin lesion developed and when there was no increase in the β-glucuronidase content in the lymph even though there was a secondary cellular and antibody response in the node. From these findings it was concluded that the increase in the level of this enzyme was associated with the development of the NLT lesion rather than with the immune response.

Variations in the Levels of Lysosomal Enzymes in the Cells of Lymph and in the Lymph Plasma During the Immune Response

The following experiments were designed to determine whether the stimulation of lymphocytes by antigen was accompanied by changes in lysosomal enzyme activity in these cells. Acid phosphatase, β-glucuronidase and N-acetyl-glucosaminidase levels were examined as examples of lysosomal enzymes and lactic dehydrogenase (LDH) was studied as an example of an intra-cellular, non-lysosomal enzyme (Figures VIII-6 and VIII-7). Immune responses to chicken erythrocytes, homologous lymphocytes, Salmonella and horse-radish peroxidase were studied. Figures VIII-6 and VIII-7 show the response to two of these antigens in terms of the total cell output in the efferent lymph, the percentage of blast cells and the activity of the various enzymes in the cells.

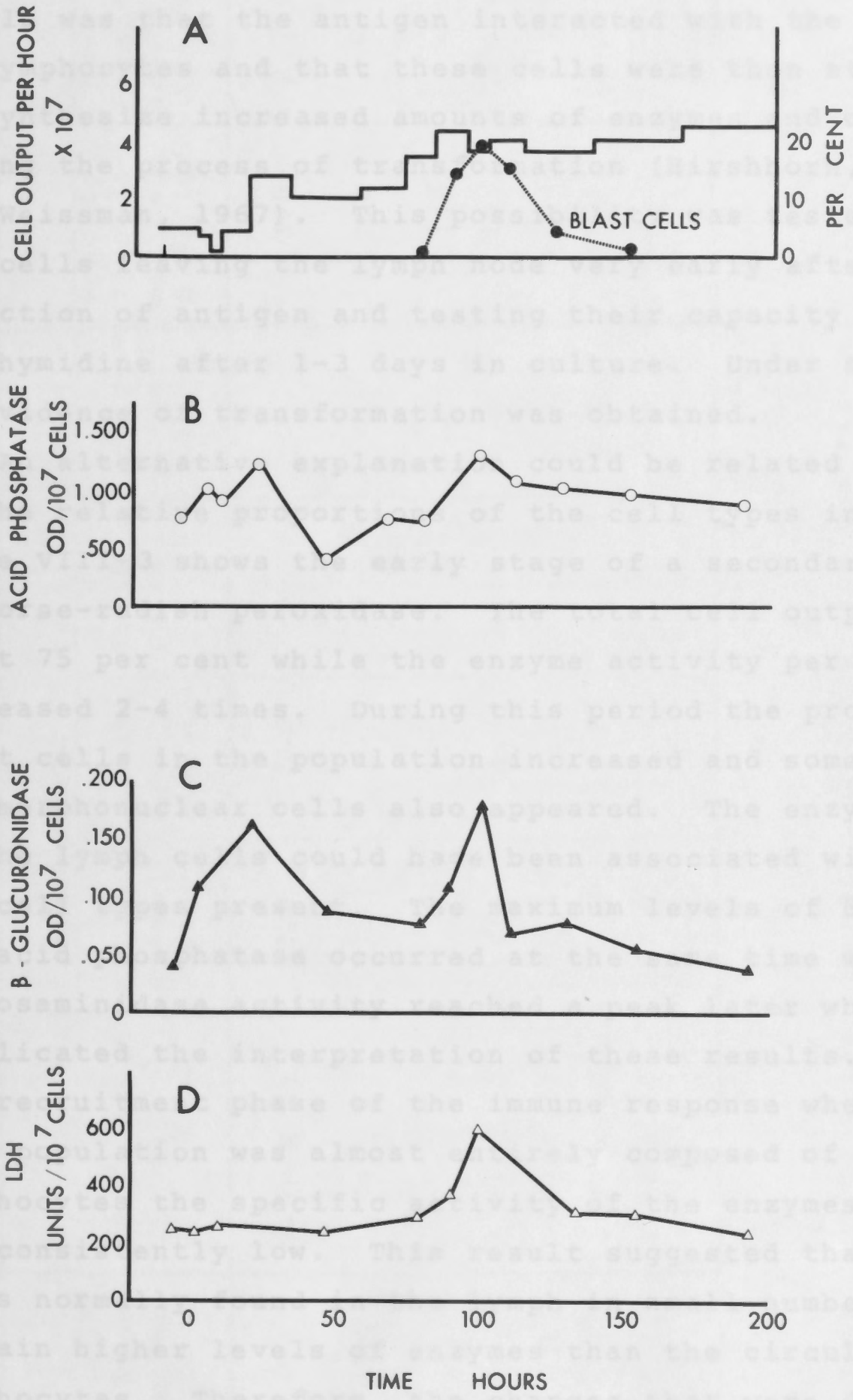
There were three distinct stages of the immune response in terms of enzyme activity. First there was a rise in the activity of the lysosomal enzymes immediately after the antigen was injected, secondly, during the recruitment phase the enzyme levels returned to near normal values and finally

FIGURE VIII - 6



The levels of glucosaminidase in efferent lymph cells during a response to Salmonella.

FIGURE VIII - 7



Enzyme levels in efferent lymph cells during an immune response to chicken erythrocytes.

an increase in the activity of these enzymes was observed during the blast cell response. These stages are now considered further.

One possible explanation for the initial rise in enzyme levels was that the antigen interacted with the population of lymphocytes and that these cells were then stimulated to synthesize increased amounts of enzymes and other proteins during the process of transformation (Hirshhorn, Hirshhorn and Weissman, 1967). This possibility was tested by collecting the cells leaving the lymph node very early after the injection of antigen and testing their capacity to incorporate ^{3}H-thymidine after 1-3 days in culture. Under these conditions no evidence of transformation was obtained.

An alternative explanation could be related to changes in the relative proportions of the cell types in the lymph. Table VIII-3 shows the early stage of a secondary response to horse-radish peroxidase. The total cell output fell by about 75 per cent while the enzyme activity per 10^{7} cells increased 2-4 times. During this period the proportion of blast cells in the population increased and some polymorphonuclear cells also appeared. The enzyme activity in the lymph cells could have been associated with any one of the cell types present. The maximum levels of β-glucuronidase and acid phosphatase occurred at the same time while the glucosaminidase activity reached a peak later which further complicated the interpretation of these results. During the recruitment phase of the immune response when the lymph cell population was almost entirely composed of circulating lymphocytes the specific activity of the enzymes in the cells was consistently low. This result suggested that other cells normally found in the lymph in small numbers probably contain higher levels of enzymes than the circulating lymphocytes. Therefore, the changes that were detected in lysosomal enzymes in the early phase of the immune response could be attributed to the changed proportion of cell types in the lymph rather than the immunological activation of small lymphocytes by antigen.

TABLE VIII-3

Time	Flow ml/hr	Cell Output hr	Differential White Cell Counts				N-Acetyl Glucosaminidase O.D./10^7	Acid Phosphatase O.D./10^7	β-Glucuronidase O.D./10^7
			Lymphocytes %	Blasts %	Neutrophils %	Eosinophils %			
1 DAY BEFORE INJECTION	4.4	9.99×10^7	95.0	5.0	0	0	0.346	0.760	-
1 HR BEFORE INJECTION	4.6	1.08×10^8	-	-	-	-	0.356	-	0.113
0-1	8.5	5.76×10^7	-	-	-	-	1.248	-	-
1-2	8.5	4.01×10^7	94.8	4.4	0.4	0.4	1.432	2.138	0.176
2-3	6.6	3.47×10^7	87.5	11.5	0.7	0.3	0.783	1.900	0.144
3-6	6.5	1.82×10^7	79.0	16.8	3.3	0.9	0.579	3.285	0.205
6-235	5.5	9.79×10^7	87.0	10.1	2.3	0.6	0.387	-	-

The lysosomal enzyme values in cells obtained from the efferent lymph during the early phase of an immune response to horse-radish peroxidase.

When the blast cells appeared the enzyme levels were consistently higher than the values obtained for normal efferent lymph cells (Figure VIII-6, VIII-7, and Table VIII-1). This result was also paralleled by the non-lysosomal enzyme, LDH, suggesting that there was an increased overall metabolic activity in the blast cells.

Only total enzyme levels have been related to the various phases of the immune response. It was noted that when the enzymes were assayed without disrupting the lysosomal membrane, values were obtained that represented about 10-20 per cent of the total values.

Discussion

Metabolism in biological systems is usually defined as the sum of; (a) the processes whereby comparatively simple chemicals are incorporated into complex biological molecules (anabolism) and (b) the processes concerned with the breakdown of complex molecules into simpler components (catabolism). Present knowledge limits the role of the lysosome strictly to catabolic processes.

Lysosomes can be defined as cytoplasmic organelles that contain hydrolytic enzymes which operate at optimum efficiency under acid pH conditions. They are membrane bound and this has led to the development of the concept of latency. That is, assays of enzyme activity in cells or in purified lysosome fractions are higher if the lysosome membrane has first been disrupted by freeze-thawing, sonication, or the action of detergents as was demonstrated in these experiments. Surface active chemicals such as retinol (vitamin A alcohol) that labilize membranes will cause the release of lysosomal enzymes from cells; agents like hydrocortisone, cholesterol, chloroquine and chlorpromazine have been shown to have the opposite effect by stabilizing the membranes of the lysosomes (Lucy, 1969).

In addition to the requirements of an acid pH for optimal activity most of these enzymes are very resistant themselves to autolysis (de Duve and Beaufay, 1959). For

this reason and because of their high molecular weight (Barrett, 1969) their transport through the lymphatic system is inevitable once they are released into the tissue fluid.

The identification of lysosomes in phagocytic cells has been well documented and the morphology of these organelles and the establishment of their biochemical activity has been described in detail. Cohn and co-workers (Cohn and Fedorko, 1969) have examined the formation of lysosomes in peritoneal macrophages *in vitro*. They measured acid hydrolases in these cells in culture and showed that the levels of these enzymes increased while the level of DNA synthesis remained constant. Inhibitors of protein synthesis depressed the formation of lysosomal enzymes (Cohn and Benson, 1965a, 1965b). Cohn and Benson, (1965c) studied by means of time-lapse cinematography, the formation of pinocytotic vesicles induced in cells cultivated in medium containing 50 per cent calf serum. More recently, macrophage differentiation has been studied by using cytochemical and autoradiographic techniques with the electron microscope (Cohn, Hirsch and Fedorko, 1966). The general conclusions of these studies suggest that in macrophages, large amounts of the plasma membrane are continually folded into the cytoplasm to form pinocytotic vesicles, some of which may contain ingested foreign material. Somehow this process stimulates the formation of new acid hydrolases which appear to be synthesized on the rough endoplasmic reticulum. After being enclosed in vesicles in the Golgi region the enzyme containing sacs fuse with the pinocytotic vesicles, liberate their contents and the process of digestion is initiated (Cohn and Fedorko, 1969). Lysosomes are termed primary if they are composed mainly of acid hydrolases, but after fusion with the pinocytozed or phagocytozed vesicle, the structure is referred to as a secondary lysosome or phagolysosome.

The phagocytic capacity of neutrophils and eosinophils has also been recognized for many years. As quoted by Vaughn (1953), as early as 1895, scientists considered the eosinophil

to be capable of phagocytosis. More recently biochemical analysis has been carried out on the nature of the granules found in these cells (Tanaka, Valentine and Fredricks, 1962; Cohn and Hirsch, 1960; Quie and Hirsch, 1964; Archer and Hirsch 1963a; Baggiolini, Hirsch and de Duve, 1969). The granules have been shown in fact, to be lysosomes and to contain high levels of acid hydrolases and other enzymes which are released into the phagocytic vacuoles and also to the exterior of the cell during phagocytosis (Hirsch, 1963; Archer and Hirsch, 1963b).

Archer (1966) has shown that eosinophils are attracted to tissues where antigen-antibody complexes are present and that complement may be involved in this process of attraction. Phagocytosis of these complexes results in granule lysis and the release of lysosomal enzymes. Although this was demonstrated *in vitro*, Movat, Uriuhara, Taichman, Rowsell and Mustard (1968), have demonstrated the phagocytosis of ferritin-antibody complexes *in vivo* by polymorphonuclear cells and platelets with a concomitant rise in the levels of lysosomal enzymes in the serum. They concluded that this increased enzyme activity originated from degranulation of the polymorphonuclear cells.

It has been suggested that lysosomes may play some role in cell division. A rearrangement and loss of lysosomes was considered by Allison and Mallucci (1964a) to precede division in cultured cells. Using euchrysine as a tracer substance Allison and Mallucci (1964b) identified structures exhibiting orange fluorescence as lysosomes in cultures of lymphocytes. In unstimulated lymphocytes 3 or 4 lysosomes were seen in the perinuclear position. It was claimed that the lysosomes became enlarged 5-24 hours after exposure to the stimulating agent. As the cells enlarged they came to contain an increased number of lysosomes which was reduced subsequently as the cells entered mitosis. Hirschhorn and Hirschhorn (1965), using PHA, showed acidophilic granules and vacuoles which contained acid phosphatase and β-glucuronidase in activated lymphocytes. They suggested

that lymphocyte activation was associated with the controlled release of lysosomal enzymes to break down existing RNA and to provide precursors for new transfer and messenger RNA. Another explanation that they considered was that the release of hydrolytic enzymes might accompany the endocytosis of PHA in transforming lymphocytes. Several other roles for lymphocyte lysosomal enzymes have been proposed. Brecher and Tanaka (1965) associated an increase in acid phosphatase activity during PHA stimulation with the transport of substances across the cell membrane rather than with a mitotic trigger. Parker, Wakasa and Lukes (1965) suggested that the lysosome in transforming lymphocytes may have a role in glycogen breakdown to provide the energy required for transformation.

The experiments described in this chapter showed an increase in lysosomal enzymes within the cells in the lymph immediately after injection of antigens. It appeared likely that the increase in enzyme activity per 10^7 lymph cells was related to the redistribution of the cell types which comprised the overall lymph population although the activation of phagocytic cells could not be ruled out. No evidence for activation of lymphocyte lysosomes in relation to cell differentiation and division was demonstrated and no specific role for the lysosome could be demonstrated in the immune response either in the lymph plasma or in the cells. The activity of β-glucuronidase in the lymph during responses to homologous lymphocytes illustrated this clearly. During a primary stimulation with homologous lymphocytes there were two distinct reactions. First there was an immune response of host origin which included the appearance of blast cells in the efferent lymph and also the production of specific antibody. The second event was a normal lymphocyte transfer reaction in which there was invasion and destruction of host tissue (Jones, Yamashita and Lafferty, 1969). The level of β-glucuronidase in the lymph coming from the NLT lesion parallelled the development of the lesion. During a secondary response there was no development of the skin lesion but there was a cellular and antibody response. No

increase in β-glucuronidase activity was found in the lymph. It was concluded therefore, that the appearance of the enzyme in the lymph plama was related to the tissue destruction associated with the NLT lesion and was not due specifically to the immune response.

During responses to other antigens, there was no significant increase in the β-glucuronidase activity in the lymph plasma except immediately after the injection of antigen. In most cases, the level of this enzyme increased in the lymph plasma but when this occurred, changes in capillary permeability associated with the inflammatory response and the appearance of polymorphonuclear cells were sufficient to explain the comparatively minor increase observed.

During the blast cell response to an antigen the lysosomal enzyme values/10^7 cells in the efferent lymph increased approximately 2 fold. However, the LDH activity in these cells also increased and therefore it seemed more reasonable to relate these increases to the overall increased metabolic activity of the blast cells rather than to specific immunological phenomena.

High levels of β-glucuronidase appeared in lymph after the injection of parasite antigens into the lower leg of sheep. There was a marked change in capillary permeability and the levels of proteins in the lymph approached those of the blood serum within about 4 hours. During this time a significant proportion of the enzyme activity in the lymph appeared to originate from the circulating blood. However, during the next 1-2 days the level of β-glucuronidase in the lymph remained high while the serum protein levels of the lymph fell. It was apparent that there was an additional source of enzyme apart from the blood and the experiments carried out <u>in vitro</u> demonstrated that the cells in the lymph, which were predominantly neutrophils and eosinophils, released lysosomal enzymes into the culture medium if both antigen and lymph (probably containing specific antibody) were present. The conclusion was reached that a significant proportion of the β-glucuronidase that was detected in the

efferent popliteal lymph during this type of inflammatory response was released from polymorphonuclear cells which had presumably phagocytozed antigen-antibody complexes.

The dramatic changes that occurred in capillary permeability may have been caused by vaso-active amines released from cells or possibly from blood platelets. While eosinophils themselves contain only small amounts of histamine (Litt, 1964), they do contain a peroxidase in their granules which is a potent histamine releasing agent which may accentuate allergic reactions (Archer, 1962).

When Vinblastin was infused directly into a lymph node there was a considerable amount of tissue destruction in the cortex of the node and a severe reduction in the cell output in the efferent lymph. The high levels of β-glucuronidase in the lymph from the lymph node treated with Vinblastin compared with the control node demonstrated that the enzyme was most likely being released from the damaged cells within the node. This experiment provided a useful control for comparing the enzyme content of the lymph in relation to tissue destruction when no inflammatory or immune response was taking place.

The involvement of lysosomal enzymes in immunological reactions appears to be confined primarily to events associated with the phagocytosis of antigen or antigen-antibody complexes.

Summary

1. Changes were recorded in the levels of lysosomal enzymes in both the cells and in the lymph plasma coming from the popliteal node after the injection of antigens.

2. When a polymorphonuclear inflammatory response was induced by the injection of parasite antigens, dramatic increases in β-glucuronidase activity resulted in the lymph. Evidence was obtained to show that some of this enzyme was released from the polymorphonuclear cells.

3. The treatment of a single popliteal node with the drug Vinblastin resulted in extensive tissue damage and the consequent release of β-glucuronidase into the lymph.

4. Lysosomal enzymes were examined during both a primary and a secondary immune response to homologous lymphocytes and it was concluded that the release of β-glucuronidase into the lymph was related to the destructive events occurring in the NLT lesion and was not due to an immune response on the part of the host.

5. Although lysosomal enzyme activity increased in the cells in lymph immediately following injection of antigen, there appeared to be no relationship between this event and the differentiation of lymphocytes.

6. Lysosomal enzyme activity in lymph cells was approximately doubled during the blast cell phase of the immune response but no specific, immunological significance was assigned to this phenomenon.

CHAPTER IX

CONCLUSIONS

Conclusions

The term immunological reaction has different connotations depending on the context in which it is used. In the biochemical sense emphasis has been placed on the production of specific immunoglobulins as the paramount feature in these reactions. To the cytologist the term no doubt suggests a range of differentiating cell types which, in a secondary sense, are involved in antibody synthesis. From a physiological viewpoint, immunological reactions, regarded in the perspective of the intact, living animal, involve a whole spectrum of interacting cellular events involving different systems and component cells. The production of antibody in this context is not necessarily an end in itself. A complete understanding of the cellular and biochemical mechanisms which are involved in the recognition of foreignness and the production of specific protein end products will not in itself describe the events which occur in the intact animal reacting to a foreign protein or microbe.

The experiments in this thesis, as well as other studies involving the role of the lymphatic system in the immune response have emphasized the dynamic character of immune reactions and the role played by the free-floating cells of lymph and blood. Whilst it has been generally appreciated that the lymphocyte is the immunologically competent cell and that this cell recirculates from the blood stream to the lymph, the magnitude of this recirculation is illustrated by the collection of these cells in gram quantities from specifically challenged lymph nodes, and from tissues where localized immune reactions are proceeding.

Antibody-forming cells themselves can exist either fixed in tissues or free-floating in the lymph and blood and evidence based on morphological characteristics, density analysis, and class of antibody produced has suggested that the cytological characteristics and synthetic capabilities of antibody forming cells may depend to some extent on the environment in which they exist.

It was shown that migratory antibody-forming cells from the lymph can have various morphological characteristics and can contain varying amounts of antibody depending on the stage of the immune response at which they appear. In this sense the spectrum of cells appearing in the lymph and in lymph nodes during an immune response appears to represent a population directed along the pathway of differentiation to plasma cells. This interpretation however, does not imply that this differentiation is an inevitable process or that ultrastructural transformation into plasma cells is incompatible with continuing DNA synthesis. It was found that antibody producing cells came in all shapes and sizes and in all stages of differentiation and that even cytologically "mature" plasma cells were still fully capable of energetic DNA synthesis. In some respects the antibody content of a cell, regardless of whether this is contained in lamellar arrangements of endoplasmic reticulum, dilated endoplasmic reticulum or in the Golgi region, provides a better description of the nature of the cell than does the term blast cell or plasma cell.

An understanding of the processes that control and regulate antibody synthesis in a single cell must be related to the general phenomena of immunological memory, synthesis of class specific antibody and probably immunological tolerance. It is possible to speculate on some of the processes that might play an important part in this regulation. In the ground state of cellular metabolism that exists in a small lymphocyte catabolic and synthetic reactions are in equilibrium. Following antigenic stimulation, the processes of differentiation or transformation in the reacting cells result from an imbalance of inducing stimuli which either promotes or restricts biochemical reactions within the cells. As the cells transform, the synthesis of RNA, DNA and structural and antibody protein increases at a rapid rate, outweighing the catabolic events. For example, the rate of RNA and DNA precursor incorporation into populations containing antibody-forming cells was increased by a factor of 4 - 20 times and

the antibody content of cells increased by an even larger amount during the immune response. In contrast, in the same populations the activity of the hydrolytic enzymes associated with catabolic processes in cells increased only by a factor of about 2.

The way in which lymphocytes are stimulated to enter into this transformation sequence is not known. Presumably antigen is the primary inducing force which in some way triggers the expression of appropriate genes within the nucleus although there is no evidence that this effect is brought about by the actual presence of antigen molecules inside the cell. In addition to this initial inductive role, there is indirect evidence to suggest that antigen continues to exert an effect even on cells that have already been induced to transform. For instance blast cells from an NLT response were more reactive when injected back into the original donor sheep than they were when injected into unrelated sheep. In contrast, Salmonella stimulated blast cells were less reactive than normal lymphocytes in giving a dermal transfer reaction. It is possible that <u>in vivo</u> the continued presence of antigen may influence an antibody-forming cell even after it has begun to synthesize antibody.

Whether genes are activated so as to lead to the formation of regulatory enzymes that control the rate of synthesis of products in antibody-forming cells is not known. One of the best studied processes of regulation in cells is the process of end-product inhibition. Excess production of certain products in a metabolic pathway has been shown to specifically inhibit the further production of products formed at an earlier stage in that particular pathway. Whether or not the end product (say antibody) is excreted or removed from the cell's environment, or partitioned into another part of the cell, could therefore be of crucial significance in regulating antibody synthesis. In terms of lymphocyte differentiation, the stock-piling or isolation of the end product, antibody, in regions of the cell distinct from the areas of protein synthesis could have important

regulatory implications and favour differentiation into the plasma cell. Thus the development of endoplasmic reticulum (ER) could serve to remove antibody, from the ribosomal areas of amino acid activation and polypeptide chain formation. The accumulation of antibody to horse-radish peroxidase was observed predominantly within the cisternae of the ER and the extent of this accumulation may be determined by the rate of entry and exit of the antibody proteins from this structure. The rate of entry of antibody into the endoplasmic reticulum may in turn be directly determined by the rate of protein synthesis on the ribosomes which in turn may be controlled by the rate of ribosomal RNA synthesis, thus completing a sequence of causes and effects initiated in some, as yet, unexplained way by an antigen.

The rate of secretion of antibody (assuming it leaves the cell via connections of the ER at the plasma membrane) could be regulated by the micro-environment of the cell. High concentrations of specific antibody in the immediate vicinity of a cell could inhibit continued antibody secretion and in the continuing presence of antigen, the formation of antigen-antibody complexes in the immediate environment of the cell may also have important regulatory functions. Some evidence was obtained which did in fact suggest that a high concentration of serum proteins external to antibody-forming cells <u>in vitro</u> inhibited the release of antibody. If mechanisms operate in the animal similar to the feedback inhibition phenomenon that has been described (Uhr and Moller, 1968) the difference between migratory antibody-forming cells in lymph and fixed antibody-forming cells in lymph nodes and tissues could be related to their different micro-environments. Inhibition of antibody release might lead to accumulation of antibody and a consequent change in the ultrastructural appearance of a cell and in this regard, the concentration of antigen or antibody immediately adjacent to the cell may be one of the critical regulatory factors.

There are differences then, between the fixed immune response as manifest by events in the node and the migratory

immune response as characterized by the cells that leave the node in the efferent lymph. In terms of the relative magnitude of these two components of the response, results were obtained which suggested that quantitatively, the contribution of antibody by the migratory cells may be equivalent to the contribution made by the fixed cells in the stimulated node. The proportion of efferent lymph cells releasing or containing antibody reached levels of 25 per cent of the total population. It seemed probable, from the effect of anti-lymphocyte serum in preventing the appearance of circulating lymphocytes in the lymph, that a population of cells could be obtained in which the majority contained detectable antibody. The use of these procedures to provide a source of concentrated and purified cellular material for biochemical studies is clearly feasible.

A further distinction can also be drawn between the fixed and migratory cells involved in immunological reactions when reactions in lymphoid organs are compared with those occurring in non-lymphoid tissues. The component cells in the reactions in non-lymphoid tissues are derived predominantly from the blood stream and migrate out of capillaries at the site where antigen or antigen-antibody complexes are localized. Once in the tissues cells can undergo the sequence of transformations that occur in lymph nodes and leads to the production of antibody. Furthermore, the phagocytic effector cells in immunological reactions can infiltrate these regions particularly when antigen-antibody reactions have occurred. Once in the tissues all of these cells depend on the lymphatic system for further transport in the body. The studies on localized NLT reactions and the acute inflammatory response emphasized the mobility of this wide range of migratory cells.

One of the most striking features of immune reactions in tissues was the extent of the cell damage and disruption to the blood vessels that occurred. The lesion produced in the NLT reaction probably is representative of lesions associated with other homograft reactions. Although specifically

reactive blast cells were isolated and specific antibody was detected, the mechanism of tissue damage could not be attributed satisfactorily to these products of the immune response. It was felt that the processes of cell transformation and proliferation alone were sufficient to cause disruption of surrounding tissue structures and a loss of functional integrity. This was probably aggravated further by the greatly increased traffic of cells through the tissue. Similarly, the traffic of polymorphonuclear cells observed during the acute inflammatory response could have contributed to the local tissue damage. These basic features of immunological reactions are overlooked in most experimental systems but from the standpoint of understanding the development of systemic immunity in the intact animal they are perhaps even more important than the knowledge of how specific antibody is synthesized.

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